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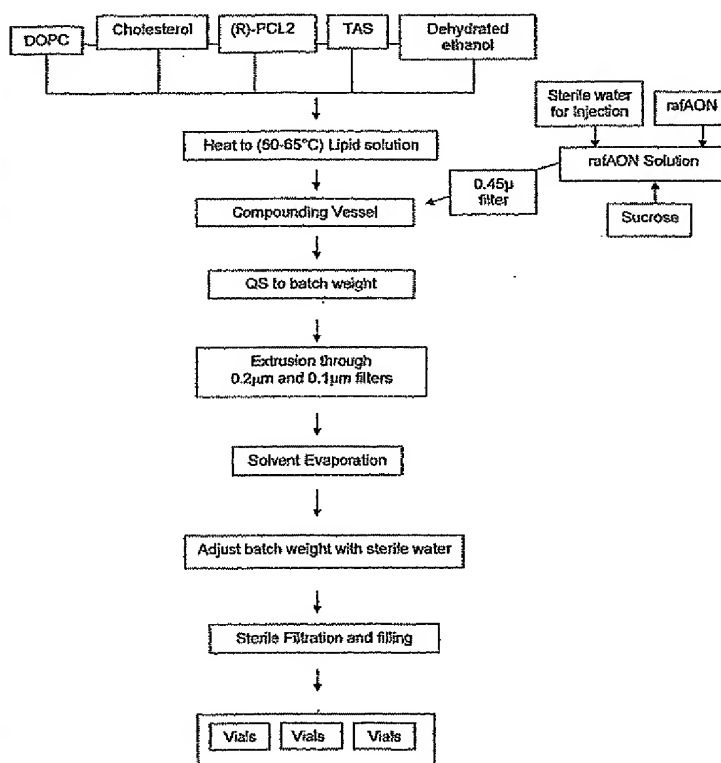
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[Continued on next page]

(54) Title: LIPID COMPOSITIONS AND USE THEREOF



(57) Abstract: The invention provides a composition suitable for use as a transfection agent, comprising a cationic cardiolipin analogue and, another lipid species. The composition of the present invention can facilitate transfection of a wide variety of polynucleotide species (e.g., oligodeoxyribonucleotides, plasmids, RNAi species, etc.). Moreover, the transfection agent of the present invention is effective in promoting transfection of primary cell cultures as well as transformed cells. Also, the inventive transfection agent is suitable for use both *in vitro* and *in vivo*. The inventive composition has additional uses as well, such as delivery of a variety of active agents, dermatological and cosmetic uses, and uses in agriculture. The invention further provides a method of introducing an active agent into a cell by contacting the cell with the inventive composition. The invention further provides a method of inhibiting the growth of neoplastic cells and a method of treating a patient suffering from a neoplastic disease by employing the inventive composition, wherein an active agent is an antineoplastic agent. The invention further provides a method for validating a genetic target, comprising (a) administering to a cell a composition comprising a cationic liposome and a siRNA, whereby the siRNA enters the cell inhibits the expression of a gene within the cell and (b)

assaying for the inhibition of the gene. The method also provides a fluorescent/ luminescent cationic cardiolipin analogue and compositions including such analogues. Using a cationic cardiolipin analogue, the invention provides a method of tracking the migration of a lipid substance within an animal.

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## LIPID COMPOSITIONS AND USE THEREOF

## CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to United States Provisional Patent Application 60/535,042, filed on January 7, 2004, the contents of which in their entirety are incorporated herein by reference thereto. This application also claims priority to United States Provisional Patent Application 60/556,843, filed on March 27, 2004, the contents of which in their entirety are incorporated herein by reference thereto. This application also claims priority to United States Provisional Patent Application 60/557,232, filed on March 29, 2004, the contents of which in their entirety are incorporated herein by reference thereto.

## FIELD OF THE INVENTION

[0002] This invention pertains to lipid compositions and their use, particularly as transfection agents.

## BACKGROUND OF THE INVENTION

[0003] Polynucleotides (such as oligonucleotides, cDNA, plasmids, RNAi, etc.) have potential use as therapeutic agents. For example, antisense oligonucleotides targeted to oncogenes have shown promise in neoplastic diseases, such as cancer. An exemplary agent, an antisense Oligonucleotide (AON) targeted to the mRNA of human c-raf oncogene, is called c-rafAON. c-rafAON is an antineoplastic agent used for the treatment of radiation resistant tumors and multiple myelomas. c-rafAON acts by hybridizing with mRNA to inhibit translation, protein synthesis and tumor growth. Other examples of polynucleotides with therapeutic benefit include RNAi that can also inhibit the expression of genes of interest, such as oncogenes. Plasmids or other vectors containing gene of interest also have therapeutic potential by being able to deliver transgenes of interest to patients.

[0004] While promising in their therapeutic potential, due to their negative charge, agents cannot efficiently penetrate cell membrane to transfect cells. Thus, a delivery system is needed to improve cell transfection and increase therapeutic efficacy of polynucleotides. The use of cationic liposomes to enhance the delivery of oligonucleotides has been reported (US Pat No. 6,559,129 B1 and 6,333,314 B1; Zelphati *et.al.* J. Liposome Res., 7(1):31-49 (1997)). However, several drawbacks exist with respect to current cationic lipid transfection agents that limit their usefulness as transfection agents.

[0005] For example, many cationic lipids have poor chemical stability. Moreover, liposomes including many cationic lipids exhibit poor loading of the polynucleotides, largely due to the inflexibility of cationic bilayer membranes. Accordingly, many currently available transfection agents are not suitable for transfection with different types of polynucleotides (DNA and RNAi). Furthermore, most available transfection agents, such as LIPOFECTIN®,

are not effective in transfecting primary cell cultures. Also, many cationic transfection agents, such as, for example, LIPOFECTIN®, exhibit relatively higher toxicity rendering them unsuitable for *in vivo* use. In light of the foregoing, there remains a need for an effective transfection agent.

[0006] A problem facing medical science is the validation of targets for genetic inhibition or therapeutic intervention. A typical method for validating a target gene as a cause or associated with a particular disorder is to ablate the gene of interest from a line of animals to generate an animal model. A common approach to accomplishing this is to generate a knock-out animal, typically a rodent. However, this approach is burdensome, costly, and time-consuming, when it works. Of course, as many genes that may be important to a particular disease also are crucial for normal development of an animal, it often is not possible to generate a knock-out. Moreover, as many genes contribute to normal physiology as well as the development of diseases, a "compensation effect" can prevent a knock-out animal from functioning as a suitable model or system for validating the target gene. Accordingly, there remains a need for an effective method of validating target genes as contributing to diseases or phenotypes.

#### BRIEF SUMMARY OF THE INVENTION

[0007] The invention provides a composition, suitable for use as a transfection agent, comprising a cationic cardiolipin analogue and, desirably, another lipid species. The composition of the present invention can facilitate transfection of a wide variety of polynucleotide species (e.g., oligodeoxyribonucleotides, plasmids, RNAi species, etc.). Moreover, the transfection agent of the present invention is effective in promoting transfection of primary cell cultures as well as transformed cells. Also, the inventive transfection agent is suitable for use both *in vitro* and *in vivo*. The inventive composition has additional uses as well, such as delivery of a variety of active agents, dermatological and cosmetic uses, and uses in agriculture.

[0008] A preferred cationic cardiolipin analogue (CCLA) is 1,3-Bis-(1,2-bis-tetradecyloxy-propyl-3-dimethylethoxyammoniumbromide)-propane-2-ol (PCL-2). The composition further preferably comprises a lipid selected from the group of lipids consisting of cholesterol, dioleoylphosphatidylethanolamine (DOPE), 1,2 Dioleoyl-sn-glycero-3-phosphocholine (DOPC), alpha tocopheryl acid succinate and any other phosphatidylcholine. The composition can include an active agent, such as a polynucleotide. The present invention also involves pharmaceutical formulations of c-raf Antisense Oligonucleotide (c-rafAON) and methods for making stable lamellar complexes of an antineoplastic agent to minimize rapid hydrolysis of raf-AON in plasma, reduce *in vivo* clearance, improve cell transfection and increase therapeutic efficacy of c-rafAON.



[0009] The invention further provides a method of introducing an active agent into a cell by contacting the cell with the inventive composition. The invention further provides a method of inhibiting the growth of neoplastic cells and a method of treating a patient suffering from a neoplastic disease by employing the inventive composition, wherein an active agent is an antineoplastic agent. The invention further provides a method for validating a genetic target, comprising (a) administering to a cell a composition comprising a cationic liposome and an RNAi, whereby the RNAi enters the cell inhibits the expression of a gene within the cell and (b) assaying for the inhibition of the gene.

[0010] The invention also provides a fluorescent cationic cardiolipin analogue and compositions including such analogues. Using a cationic cardiolipin analogue, the invention provides a method of tracking the migration of a lipid substance within an animal. This invention further provides a luminescent cationic cardiolipin analogue and composition including such analogues that will be helpful in measurements requiring higher decay time than fluorescent analogues.

[0011] These and other advantages of the invention, as well as additional inventive features, will be apparent from the description of the invention provided herein.

#### BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Figure 1 depicts the manufacturing process for LEafAON-ETU.

[0013] Figure 4A graphically presents data concerning the therapeutic efficacy of DDAB-Liposome or Cationic Cardiolipin Liposome-Based Antisense Oligonucleotide (AON) in combination with Taxotere® against human prostate (PC-3) tumor growth in SCID mice.

[0014] Figure 4B depicts the effect of DDAB-Liposome or Cationic Cardiolipin Liposome-Based Antisense Oligonucleotide (AON) in combination with Taxotere® against human prostate (PC-3) tumor in SCID Mice.

[0015] Figure 4C graphically presents data concerning the toxicity of DDAB\*-Liposome or Cationic Cardiolipin Liposome-Based Antisense Oligonucleotide (AON) in Mice.

[0016] Figure 5A graphically presents data concerning the transfection efficiency of the NeoPhectin™ in CHO cells.

[0017] Figure 5B graphically presents data concerning the transfection efficiency of the NeoPhectin™ in COS-1 cells.

[0018] Figure 5C graphically presents data concerning NeoPhectin™ mediated delivery of RNAi in Primary Rat Lung Microvessel Endothelial Cells (RLMVEC).

[0019] Figure 5D graphically presents data concerning the transfection efficiency of the NeoPhectin™ in BALB/3T3 cells.

[0020] Figure 5E graphically presents data concerning the inhibition of a cytokine receptor (TbRII) expression with dsRNA interference using NeoPhectin™ in Rat Pulmonary Microvessel Endothelial Primary Cell Culture (PLMVEC).

[0021] Figure 5F depicts the effect of NeoPhectin™ mediated delivery of RNAi (200nM) oligonucleotides in primary cells after 48 hours.

[0022] Figure 5G graphically presents data demonstrating that the NeoPhectin™ (2.5 µg/mL) mediated delivery of RNAi oligonucleotides inhibits the T\_RII Gene in Primary Human Umbilical Vein Endothelial Cells (HUVEC).

[0023] Figure 5H graphically presents data demonstrating that the NeoPhectin™ (2.5 µg/mL) mediated delivery of RNAi oligonucleotides inhibits the T\_RII Gene in Primary Human Pulmonary Artery Endothelial Cells (HPAEC).

[0024] Figure 5I graphically presents data demonstrating that the NeoPhectin™ (2.5 µg/mL) mediated delivery of RNAi oligonucleotides inhibits the T\_RII Gene in Primary Rat Lung Microvessel Endothelial Cells (RLMVEC).

[0025] Figure 6A graphically presents data concerning the transfection efficiency of PCL-2-(CCLA)-based liposomes in CHO, BALB/3T3, HepG<sub>2</sub>, MX-1, and A549 cells.

[0026] Figure 6B graphically presents data concerning the transfection activity of PCL-2-CCLA-based liposomes in the lung (top panel) and heart (bottom panel) tissue of Balb/c mice.

[0027] Figure 6C graphically presents data concerning the transformation efficiency of CCLA-based liposomes compared to that of *In Vivo* GeneSHUTTLE™ in Balb/c mice.

[0028] Figure 6D graphically presents data concerning the *in vitro* efficacy of siRNA delivered by CCLA-based liposome against c-raf in A549, PC-3, MDA-MB-231, and SK-OV-3 cancer cells.

[0029] Figure 6E graphically presents data concerning the *in vivo* efficacy of siRNA delivered by CCLA-based liposome against c-raf using the human breast cancer tumor xenograft model in SCID mice.

[0030] Figure 7A depicts Raf-1 expression in animals treated with rafsiRNA-NeoPhectin™ in the human prostate tumor model (PC-3) in CB-17 SCID mice.

[0031] Figure 7B graphically presents data concerning Raf-1 expression in animals treated with rafsiRNA-NeoPhectin™ in the human prostate tumor model (PC-3) in CB-17 SCID mice.

[0032] Figure 7C depicts Raf-1 expression in animals treated with rafsiRNA-NeoPhectin™ and Taxotere® in the human prostate tumor model (PC-3) in CB-17 SCID mice.

[0033] Figure 7D graphically presents data concerning Raf-1 expression in animals treated with rafsiRNA-NeoPhectin™ and Taxotere® in the human prostate tumor model (PC-3) in CB-17 SCID mice.

[0034] Figure 7E depicts cyclin D-1 expression in animals treated with rafsiRNA-NeoPhectin™ in the human prostate tumor model (PC-3) in CB-17 SCID mice.

[0035] Figure 7F graphically presents data concerning cyclin D-1 expression in animals treated with rafsiRNA-NeoPhectin™ in the human prostate tumor model (PC-3) in CB-17 SCID mice.

[0036] Figure 7G depicts cyclin D-1 expression in animals treated with rafsiRNA-NeoPhectin™ and Taxotere® in the human prostate tumor model (PC-3) in CB-17 SCID mice.

[0037] Figure 7H graphically presents data concerning cyclin D-1 expression in animals treated with rafsiRNA-NeoPhectin™ and Taxotere® in the human prostate tumor model (PC-3) in CB-17 SCID mice.

[0038] Figure 7I graphically presents data concerning the ability of rafsiRNA-NeoPhectin™ to inhibit tumor growth and improve response to Taxotere® in PC-3 tumor xenograft CB-17 SCID mice.

[0039] Figures 8A, 8B, and 8C depict synthesis of cationic cardiolipin molecules useful in the context of the present invention.

#### DETAILED DESCRIPTION OF THE INVENTION

[0040] In one embodiment, the invention provides a composition, comprising a cationic cardiolipin analogue (CCLA) and, desirably, another lipid species. Suitable cationic cardiolipin analogues for use in the inventive composition are described in international patent application PCT/US03/33099, the disclosure of which is incorporated herein in its entirety by reference thereto. A most preferred cationic cardiolipin analogue (CCLA) for inclusion in the inventive composition is 1,3-Bis-(1,2-bis-tetradecyloxy-propyl-3-dimethylethoxyammoniumbromide)-propane-2-ol (PCL-2), which is identified in PCT/US03/33099 as compound 19.

[0041] The invention also provides fluorescent and/or luminescent cationic cardiolipin analogues. For example, the fluorescent analogue can comprise a cationic cardiolipin molecule (such as described in international patent application PCT/US03/33099) as compound 19 conjugated to a fluorescent moiety. Examples of fluorescent moieties includes fluorescein derivatives such as [N-(5-fluoresceinyl)-5-carbamylpentanoic acid] and the like, 2,1,3-benzooxadiazole derivatives, coumarin derivatives such as 3-bromoacetyl-6,7-methylenedioxycoumarin, 4-(7-diethylaminocoumarin-3-yl) benzeneisocyanate and the like, 4-(4,5-diphenyl-1H-imidazo-2-yl) benzoyl chloride, 2-dansylethyl chloroformate, 5-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride and the like. A preferred fluorescent analogue is a fluorescent analogue of PCL-2. The fluorescent cationic cardiolipin analogue can be constructed by any suitable method for conjugating fluorescent moieties to molecules such as cationic lipids, many of which are known to those of ordinary skill in the art. Luminescent analogues can be also synthesized

by ordinary skill in the art. The fluorescent or luminescent analogues can be employed in the invention.

**[0042]** Fluorescent and luminescent cationic cardiolipin of the present invention can be used to track the path/ location of cationic cardiolipin liposomes. Fluorescent and luminescent cationic cardiolipin of the present invention also can be used to investigate the distribution of liposome uptake and gene delivery in various tissues. Also, fluorescent and luminescent cationic cardiolipin of the present invention can be used to investigate the mechanism of delivery of genes inside the cell.

**[0043]** As noted herein, any suitable amount of cationic cardiolipin analogue (CCLA) can be used in the composition, desirably, the cationic cardiolipin analogue PCL-2 is present at between about 0.3 mM and about 20 mM, such as between about 1 mM and about 15 mM, but the composition can include more or less cationic cardiolipin PCL-2 as desired. In certain preferred compositions, the cationic cardiolipin PCL-2 is present at about 0.35 mM.

**[0044]** Desirably, the composition further comprises at least one lipid in addition to the cationic cardiolipin analogue. The additional lipid(s) can be any desired lipid species suitable for forming the composition of interest, such as those described in PCT/US03/33099.

Preferred lipids for inclusion in the inventive composition are selected from the group of lipids consisting of cholesterol, cholesterol derivatives, dioleoylphosphatidylethanolamine (DOPE), 1,2 Dioleoyl-sn-glycero-3-phosphocholine (DOPC), alpha tocopheryl acid succinate and any other phosphatidylcholine. Typically, the total lipid concentration of the composition is between about 1.0 mg/mL and about 60 mg/mL (such as at least about 5.0 mg/mL or at least about 10 mg/mL or even at least about 10 mg/mL and up to about 50 mg/mL, or up to about 40 mg/mL or even up to about 30 mg/mL) but can be higher or lower than these amounts as desired.

**[0045]** A most preferred composition in accordance with the present invention includes DOPC, cationic cardiolipin PCL-2, and cholesterol. In this composition, the cationic cardiolipin analogue, DOPC, and cholesterol can be present in any suitable proportion. However, such a composition preferably has a percent molar ratio of DOPC:PCL-2:cholesterol of between about (50-65):(25-35):(5-20). Also, desirably such a composition further includes D-alpha tocopheryl acid succinate. The D-alpha tocopheryl acid succinate can be present in any suitable amount in the composition, but desirably is present at between about 0.1 wt % and about 1 wt %, most preferably at about 0.2 wt%. However, the amount of D-alpha tocopheryl acid succinate can be more or less than these amounts, as desired.

**[0046]** Another preferred composition according to the invention includes cationic cardiolipin PCL-2 and cholesterol. Within such a composition, the cationic cardiolipin and cholesterol can be present in any suitable molar ratio. However, desirably the molar ratio of

PCL-2 and cholesterol is between 1:3 and 6:1, such as between 1:3 and 3:1, or between 2:3 and 3:2, or even between 1:2 and 2:1.

[0047] Another preferred composition according to the invention includes PCL-2 and DOPE. Within such a composition, the cationic cardiolipin and DOPE can be present in any suitable molar ratio. However, desirably the molar ratio of PCL-2 and DOPE is between 1:3 and 6:1, such as between 1:3 and 3:1, or between 2:3 and 3:2, or even between 1:2 and 2:1.

[0048] In addition to the cationic cardiolipin and other lipids, the composition can include stabilizers, absorption enhancers, antioxidants, phospholipids, biodegradable polymers and medicinally active agents among other ingredients. Suitable antioxidants include compounds such as ascorbic acid, tocopherol, and dextroxime mesylate. Suitable absorption enhancers include Na-salicylate-chenodeoxy cholate, Na deoxycholate, polyoxyethylene 9-lauryl ether, chenodeoxy cholate-deoxycholate and polyoxyethylene 9-lauryl ether, monolein, Na tauro-24,25-dihydrofusidate, Na taurodeoxycholate, Na glycochenodeoxycholate, oleic acid, linoleic acid, and linolenic acid. Polymeric absorption enhancers can also be included such as polyoxyethylene ethers, polyoxyethylene sorbitan esters, polyoxyethylene 10-lauryl ether, polyoxyethylene 16-lauryl ether, and azone (1-dodecylazacycloheptane-2-one).

[0049] In some embodiments, it is preferable for the inventive composition, especially liposomal composition to include a targeting agent, such as carbohydrate or a protein or other ligand that binds to a specific substrate, such as antibodies (or fragments thereof) or ligand that recognize cellular receptors. The inclusion of such agents (such as a carbohydrate or one or more proteins selected from groups of proteins consisting of antibodies, antibody fragments, peptides, peptide hormones, receptor ligands such as an antibody to a cellular receptor and mixtures thereof) can facilitate targeting the composition to a predetermined tissue or cell type.

[0050] In a preferred embodiment, the composition includes at least one sugar or biodegradable polymer. Examples of suitable sugars and polymers include sucrose, lactose, trehalose, dextrose, epichlorohydrin, branched hydrophilic polymers of sucrose, polyethylene glycols, polyvinyl alcohols, methoxypolyethylene glycol, ethoxypolyethylene glycol, polyethylene oxide, polyoxyethylene, polyoxypropylene, polyvinylpyrrolidone, polypyrrolidone, dextran, cellulose acetate, sodium alginate, N, N-diethylaminoacetate, block copolymers of polyoxyethylene and polyoxypropylene, polyvinyl pyrrolidone, polyoxyethylene X-lauryl ether wherein X is from 9 to 20, and polyoxyethylene sorbitan esters. A preferred sugar for inclusion in the inventive composition is sucrose. Where the sugar or polymer is present, it typically represents between about 1 wt% and about 20 wt% of the composition, such as between about 5 wt% and about 30 wt%. Especially where the sugar is sucrose, a suitable amount of sugar for inclusion in the composition is 10-12 wt%. Additionally, the composition also can include carriers, such as physiologically acceptable carriers. Suitable

carriers for physiological (e.g., medical, veterinary, experimental, etc.) uses are physiologically-compatible buffers (e.g., phosphate-buffered saline (PBS), HEPES, etc.), many of which are known to those of ordinary skill in the art.

**[0051]** The composition typically has a pH of between about 3 and about 8. However, the pH of the composition can vary considerably depending on its desired use. It is within the ordinary skill of the art to select a suitable pH for a desired use. Thus, in some embodiments, the pH of the composition can be adjusted to be acidic (e.g., between about 2 and about 6.9, such as between about 3 and about 6 or between about 4 and about 5). In other applications, it is desirable for the pH of the composition to be alkaline (e.g., between about 7.1 and about 11, or between about 8 and about 10, such as about 9). The pH of the composition can be adjusted using suitable acidic or alkaline buffers as are known to those of ordinary skill in the art.

**[0052]** The composition can be of any suitable form, such as liposomal formulations, complexes, emulsions, suspensions, etc. Such formulations can be prepared by any suitable technique, depending on the type of composition, which are known to those of ordinary skill in the art. A preferred composition is a liposomal composition or other composition containing lipid vesicles. Such composition can include unilamellar or multilamellar vesicles, or mixtures thereof. Any suitable technique can be employed to produce such a liposomal formulation. Suitable techniques include the thin-film hydration method, reverse phase evaporation, ethanol injection, etc. For example, lipophilic liposome-forming ingredients can be dissolved or dispersed in a suitable solvent or combination of solvents and dried. Suitable solvents include any non-polar or slightly polar solvent, such as *t*-butanol, ethanol, methanol, chloroform, or acetone that can be evaporated without leaving a pharmaceutically unacceptable residue. Drying can be by any suitable means such as lyophilization, use of a rotary dryer, etc. Hydrophilic ingredients, such as some pharmaceutical agents, preservatives, and other agents, can be dissolved in polar solvents, including water, which can be mixed with the lipid phase either prior to drying or upon reconstitution. Mixing the dried lipophilic ingredients with the hydrophilic mixture can form liposomes. Mixing the polar solution with the dry lipid film can be by any means that strongly homogenizes the mixture. Vortexing, magnetic stirring and/or sonicating can effect the homogenization.

**[0053]** Where active agents are included in the liposomes they can be dissolved or dispersed in a suitable solvent and added to the liposome mixture prior to mixing. Typically hydrophilic active agents will be added directly to the polar solvent and hydrophobic active agents will be added to the nonpolar solvent used to dissolve the other ingredients but this is not required. The active agent could be dissolved in a third solvent or solvent mixture and added to the mixture of polar solvent with the lipid film prior to homogenizing the mixture.

[0054] The liposomes of the present invention can be multi or unilamellar vesicles depending on the particular composition and procedure to make them. Liposomes can be prepared to have substantially homogeneous sizes in a selected size range, such as about 1 micron or less, or about 500 nm or less, about 200 nm or less, or about 100 nm or less. Particle size has been shown to play a major role in liposome biodistribution and the route of cell entry. Larger liposomes are distributed primarily to the reticuloendothelial (RES) system with negligible amounts in other tissues, whereas smaller liposomes are localized to other organs. Additionally, the clearance of multilamellar vesicles of heterogenous size distribution follows a biphasic pattern, with rapid clearance of larger liposomes and a slow rate of clearance of small liposomes. Limited information is available on the biodistribution of cationic liposomes containing oligonucleotides. Letsinger and colleagues previously reported that oligonucleotides complexed with cationic liposomes, approximately 2.0 microns in diameter, are transiently taken up by the lungs followed by rapid distribution to liver. Recent studies demonstrated that endocytosis is the principal pathway for delivery of oligonucleotides via cationic liposomes. Preferably, the liposomal lamellar complex formulation of the present invention includes small liposomes so as to slow the rate of clearance of the active agent. One effective sizing method involves extruding an aqueous suspension of the liposomes through a series of polycarbonate membranes having a selected uniform pore size; the pore size of the membrane will correspond roughly with the largest sizes of liposomes produced by extrusion through that membrane. For physiological use, it is preferable for the liposomes to be extruded through 0.22  $\mu\text{m}$  filters or less to effect sterilization of the formulation. Desirably, the mean size of the liposomes is between about 50 nm and about 250 nm, such as between about 100 nm and about 200 nm or between about 150 nm and 250 nm. Preferably, the size distribution of the liposomes is substantially uniform. Moreover, within a preparation, desirably, about 99% of the liposomes have a diameter less than about 500 nm (i.e., D99 less than about 500 nm), and desirably, the D99 of a liposomal composition according to the invention is between about 170 nm and about 500 nm, such as between about 200 nm and about 350 nm or between about 250 nm and about 300 nm).

[0055] A preferred manufacturing protocol involves dissolving: i) the lipid excipients, namely, DOPC, cholesterol, PCL-2 and D-Alpha tocopheryl acid succinate in dehydrated ethanol and ii) the active ingredient – a polynucleotide (indicated as a c-raf-1 antisense oligonucleotide in Figure 1), and sucrose in sterile water for injection. Ethanolic lipid solution is added to aqueous active solution to form liposomes. Following *quantum sufficit* (QS) to batch weight according to which the product weight is brought up to the desired weight depending on the batch size, liposomes are size reduced by extrusion three times through 0.2 $\mu$  pore size polycarbonate membrane filters and five times through 0.1 $\mu$  pore size



polycarbonate membrane filters to meet size specification. . Ethanol added is removed from extruded liposomes by rotary evaporation under vacuum. After adjusting the product weight to the weight prior to solvent removal with sterile water for injection, the product is filtered through 0.22 $\mu$  sterilizing filter, filled in sterile vials, stoppered and sealed. This process is used to manufacture the formulation at 10-20 kilogram scale.

[0056] It has been found that the composition of the present invention is stable. In this sense, the composition can be stored under refrigeration and at room temperature for an extended period of time. Generally, the composition is stable under such conditions for at least about 2 months. Stability can be assessed by measuring a change in the constitution of the composition over a desired time frame. For example, the composition can be considered stable if, after two months storage at either room temperature or refrigeration, the composition includes at least about 70-110% of the amount of cationic cardiolipin (e.g., PCL-2) present when initially formulated. A formulation is considered stable when no more than 10% of the active moiety is lost during the shelf life of the product.

[0057] The liposomal (or other lipid) composition or formulation can be in any desired form. For example, for pharmaceutical use, the composition can be ready for administration to a patient. Where such compositions contain liposomes or other types of lipid vesicles, such formulations typically are in the form of vesicles in an aqueous medium (such as ethanol and water for injection). Alternatively, the formulation can be in dried or lyophilized form, in which instance, the composition preferably includes a cryoprotectant as well. Suitable cryoprotectants include, for example, sugars such as trehalose, maltose, lactose, sucrose, glucose, and dextran, with the most preferred sugars from a performance point of view being trehalose and sucrose. Other more complicated sugars can also be used, such as, for example, aminoglycosides, including streptomycin and dihydrostreptomycin.

[0058] The composition according to the present invention including the inventive cationic cardiolipin can be employed in a variety of applications, such as the *in vivo* or *in vitro* delivery of active agents such as peptides, polypeptides, proteins, nucleotides, polynucleotides, small molecules, or other active agents to human or animal (typically vertebrate) patients, to plants, or to cells in culture. To facilitate such uses, the composition can, in addition to the cationic cardiolipin species, other lipids, and other constituents, further include at least one such active agent. The compositions can be used, for example, in any procedure comprising the use of liposomes or lipid vesicles to deliver substances intracellularly either *in vitro* or *in vivo* (Felgner *et al.* US patent no 5,264,618). The compositions also can be used cosmetically, for example, as a dermatological preparation. Accordingly, the composition can be formulated for use depending on the desired end use.

[0059] A most preferred active agent for inclusion within the inventive composition is a polynucleotide, which can be DNA, RNA, or mixtures of DNA and RNA. Examples of



therapeutic polynucleotides include ribozymes, interfering RNA (RNAi) antisense RNA or DNA sequences, which can target desired sequences within cells, such as genes associated with a disease state (e.g., oncogenes or viral genes). Desirably, the polynucleotide for use in the inventive composition hybridizes to a specific human mRNA within cells and further inhibits gene expression as a result of hybridization to the targeted mRNA. For example, the polynucleotide can be targeted to an oncogene such as ras, raf cot, mos, myc, etc., and preferably is targeted to the human c-raf gene. Other preferred genes for targeting with therapeutic ribozymes, interfering RNA (RNAi) antisense RNA or DNA sequences include viral genes, particularly HIV genes, such as the rev transactivator. In other embodiments, a therapeutic polynucleotide can be one which is absent or mutated in a diseased state, or can encode a gene product that is deficient or absent in a diseased state. Other polynucleotides can encode therapeutic polypeptides, such as, for example, immunogenic peptides (which can be used as vaccines), natural hormones, or a synthetic analogue of a natural hormone.

**[0060]** A preferred polynucleotide for use in the inventive composition is a 5 to 50-mer antisense oligodeoxyribonucleotide, preferably a 10-40-mer sequence, and more preferably a 15-25-mer sequence targeted to a specific gene of interest (see, e.g., U.S. Patent 6,126,965, disclosing a 15-mer anti c-raf-1 oligonucleotide having the sequence 5'-GTGCTCCATTGATGC-3'). Additional suitable anti-raf oligodeoxyribonucleotide sequences are known in the art and can be suitably used in the context of the present invention (see, e.g., U.S. Patents 6,090,626, 6,126,965, 6,333,314, 6,410,518 and international patent application publications WO 94/15645 and 94/23755). Where oligonucleotides are included in the composition, they preferably contain one or more phosphothioate linkages preferably two phosphothioate linkages. Most preferably, oligonucleotides for inclusion in the inventive composition contain one phosphothioate linkage at each terminal end, but they can be present anywhere from one end to the other end (e.g., between the ends) of an oligonucleotide.

**[0061]** A most preferred composition according to the invention contains a 15-mer anti c-raf-1 oligonucleotide having the sequence 5'-GTGCTCCATTGATGC-3', 1,2-dioleoyl-sn-Glycero-3-Phosphocholine (DOPC), novel positively charged phospholipids 1,3-Bis-(1,2-bis-tetradecyloxy-propyl-3-dimethylethoxyammoniumbromide)-propane-2-ol (PCL-2) and cholesterol as stabilizing agents, D-alpha tocopheryl acid succinate as an antioxidant, sucrose as a tonicity agent and ethanol and water for injection as solvents. This composition can be provided as a sterile liquid containing 2 mg/mL rafAON (50 mg rafAON per vial). Each vial is diluted 2-fold with 5% dextrose, USP to yield a 1 mg/mL rafAON liposome formulation. It may be further diluted up to 8-fold with 5% dextrose, USP prior to administration.

**[0062]** Another preferred polynucleotide for use in the inventive composition is an RNAi species. RNAi technology provides an effective method of suppressing the activity of cellular genes. siRNA (RNAi) is an RNA molecule having a double-stranded structure (dsRNA) comprising a first complimentary strand and second complimentary strand. The double stranded RNA comprises about 20-26 nucleotides in length, preferably 21 or 23 nucleotides. Each of the first two strands of the dsRNA has a 5' terminus and 3' terminus. Each strand of dsRNA has 1-4 nucleotide hangovers, preferably 2 nucleotides on the 3' terminus. The hangover nucleotides typically comprises thymidine or uridine. Within the siRNA, The first strand of double stranded RNA (dsRNA) comprises nucleotide sequence corresponding at least part of its sequence to the target sequence (e.g., a target mRNA transcript portion of the target gene). The second strand of dsRNA is complimentary to the first strand of dsRNA. Desirably, the target sequence corresponds to an oncogene, such as those discussed herein (preferably c-raf-1), but can be any desired target gene. It is within the ordinary skill in the art to construct a suitable RNAi for targeting a particular gene. Desirably, the dsRNA is produced by chemical synthesis.

**[0063]** The gene to be targeted with RNAi in accordance with the invention can be selected from any desired species, depending on the desired end application. Thus, for example, the composition can find agricultural use by targeting genes in plants, such as crop plants, or the genes of plant or fungal parasites or pathogens. Similarly, the RNAi can be targeted to animal genes, such as those of pets (e.g., cats, dogs, etc.), common livestock species (e.g., cattle, pigs, sheep, goats, poultry (such as chickens, turkeys, or game fowl), or fur-bearing animals such as mink, sable, fox, and the like. The gene to be targeted also can be of a parasite or pathogen (e.g., bacteria or viral pathogen) of such species.

**[0064]** The technology also will find medical application by targeting human genes. In a preferred embodiment, the gene to be targeted (e.g., with oligonucleotides or siRNA) is a human or animal oncogene, such as ras, raf cot, mos, myc, and the like. Most preferably, the gene to be targeted is c-raf-1. The sequences of such genes are published, and those of skill in the art are able to construct siRNA that target such genes without undue experimentation. Guidance in this respect is additionally supplied by success in suppressing such genes by antisense RNA and oligonucleotides. Examples of siRNA molecules for use in the context of the present invention include:

SEQ ID NO: 1

5'-UGGAAUGAGCUUACAUGACdTdT-3'

3'-dTdTACCUUACUCGAAUGUACUG-5'

## SEQ ID NO: 2

5'-GCACGCUUAGAUUGGAACAdTdT-3'  
3'-dTdTTCGUGCGAAUCUAACCUUGU-5'

## SEQ ID NO: 3

5'-UGCGUCGGAUGCGAGAAUCdTdT-3'  
3'-dTdTACGCAGCCUACGCUCUUAG-5'

## SEQ ID NO: 4

5'-UCCGGAUGCAGGAUGACAAdTdT-3'  
3'-dTdTAGGCCUACGUCCUACUGUU-5'

## SEQ ID NO: 5

5'-UCAACAGGAGCGCCUCUGAdTdT-3'  
3'-dTdTAGUUGUCCUCGCGGAGACU-5'

## SEQ ID NO: 6

5'-UGCUUGCACGCUGACUACAdTdT-3'  
3'-dTdTACGAACGUGCGACUGAUGU-5'

## SEQ ID NO: 7

5'-AUUCCUGCUCAAUGGAUUUdTdT-3'  
3'-dTdTUAAGGACGAGUUACCUAAA-5'

## SEQ ID NO: 8

5'-GCUGCAUCAAUGGAGCACAdTdT-3'  
3'-dTdTTCGACGUAGUUACCUCGUGU-5'

## SEQ ID NO: 9

5'-CUGCAUCAAUGGAGCACAUdTdT-3'  
3'-dTdTGACGUAGUUACCUCGUGUA-5'

## SEQ ID NO: 10

5'-UGCAUCAAUGGAGCACAUAdTdT-3'  
3'-dTdTACGUAGUUACCUCGUGUAU-5'

## SEQ ID NO: 11

5'-GCAUCAAUGGAGCACAUACdTdT-3'  
3'-dTdTTCGUAGUUACCUCGUGUAUG-5'

SEQ ID NO: 12

5'-GCUGCAUCAAUGGAGCACAUU-3'  
3'-UUCGACGUAGUUACCUCGUGU-5'

SEQ ID NO: 13

5'-CUGCAUCAAUGGAGCACAUUU-3'  
3'-UUGACGUAGUUACCUCGUGUA-5'

SEQ ID NO: 14

5'-UGCAUCAAUGGAGCACAUUU-3'  
3'-UUACGUAGUUACCUCGUGUAU-5'

SEQ ID NO: 15

5'-GCAUCAAUGGAGCACAUACUU-3'  
3'-UUCGUAGUUACCUCGUGUAUG-5'

SEQ ID NO: 16

5'-GCUGCAUCAAUGGAGCACAUAdTdT-3'  
3'-dTdTTCGACGUAGUUACCUCGUGUAU-5'

SEQ ID NO: 17

5'-AAGCUGCAUCAAUGGAGCACAdTdT-3'  
3'-dTdTUUCGACGUAGUUACCUCGUGU-5'

SEQ ID NO: 18

5'-GCAUCAAUGGAGCACAUACAGdTdT-3'  
3'-dTdTTCGACGUAGUUACCUCGUGUAUGUC-5'

SEQ ID NO: 19

5'-CUGCAUCAAUGGAGCACAUACdTdT-3'  
3'-dTdTTCGACGUAGUUACCUCGUGUAUG-5'

SEQ ID NO: 20

5'-AAGCUGCAUCAAUGGAGCACAUACdTdT-3'  
3'-dTdTUUCGACGUAGUUACCUCGUGUAUG-5'

SEQ ID NO: 21

5'-GCUGCAUCAAUGGAGCACAUACAGdTdT-3'  
3'-dTdTTCGACGUAGUUACCUCGUGUAUGUC-5'

SEQ ID NO: 22

5'-GCAUCAAAUGGAGCACAUACAGGGAdTdT-3'  
3'-dTdTTCGUAGUUACCUCGUGUAUGUCCCU-5'

SEQ ID NO: 23

5'-UUAAGCUGCAUCAAAUGGAGCACAUdTdT-3'  
3'-dTdTAAUUCGACGUAGUUACCUCGUGUA-5'

SEQ ID NO: 24

5'-GCUGCAUCAAAUGGAGCACAUUU-3'  
3'-UUCGACGUAGUUACCUCGUGUAU-5'

SEQ ID NO: 25

5'-AAGCUGCAUCAAAUGGAGCACAUU-3'  
3'-UUUUCGACGUAGUUACCUCGUGU-5'

2SEQ ID NO: 26

5'-GCAUCAAAUGGAGCACAUACAGUU-3'  
3'-UUCGUAGUUACCUCGUGUAUGUC-5'

SEQ ID NO: 27

5'-CUGCAUCAAAUGGAGCACAUACUU-3'  
3'-UUGACGUAGUUACCUCGUGUAUG-5'

SEQ ID NO: 28

5'-AAGCUGCAUCAAAUGGAGCACAUACUU-3'  
3'-UUUUCGACGUAGUUACCUCGUGUAUG-5'

SEQ ID NO: 29

5'-GCUGCAUCAAAUGGAGCACAUACAGUU-3'  
3'-UUCGACGUAGUUACCUCGUGUAUGUC-5'

SEQ ID NO: 30

5'-GCAUCAAAUGGAGCACAUACAGGGAUU-3'  
3'-UUCGUAGUUACCUCGUGUAUGUCCCU-5'

SEQ ID NO: 31

5'-UUAAGCUGCAUCAAAUGGAGCACAUUU-3'  
3'-UUAUUCGACGUAGUUACCUCGUGUA-5'

[0065] In some embodiments, it is preferred for the composition to comprise a plurality of active agents, such as those identified herein and otherwise known to those of skill in the art. For example, while the inventive composition desirably includes at least one nucleic acid species as noted above (e.g., oligonucleotide, dsRNA, etc.), the composition can include more than one such species. For example, the inventive composition can include a plurality of oligonucleotides, siRNA species, or mixtures thereof. In this respect, in a preferred embodiment, at least two active agents present within the inventive composition comprise nucleic acids. For example, at least one of said nucleic acids can be an siRNA species, an oligonucleotide, or another species of nucleic acid, as desired. Where a nucleic acid targets a gene sequence (e.g., for inhibition, such as RNAi or antisense oligonucleotide inhibition), a first and a second nucleic acid species present in the composition can be designed to target separate genetic sequences. Such separate target sequences can be found in separate genes, which can facilitate inhibiting multiple genes in target cells. Alternately or in addition, the separate target sequences can be found in the same gene. In such embodiments, the combined effect of the different species of inhibiting nucleic acids in the composition can enhance the ability to reduce expression of the target gene.

[0066] Desirably, where polynucleotides are included within the inventive composition, the respective charge ratio of cationic lipid: polynucleotide (e.g., oligodeoxyribonucleotide, RNAi, etc.) ranges from (1-4):1, such as between 2:1 and 3:1. Because PCL-2 has two positive charges, the overall lipid:drug molar amounts can be lower in the present invention than in previously reported cationic liposomal formulations. Desirably the respective total lipids to polynucleotide (e.g., oligodeoxyribonucleotide, RNAi, etc.) molar amounts are between about 10:1 and about 200:1, such as between about 20:1 and about 150:1 or between about 50:1 and about 100:1. Where the composition is liposomal, desirably at least about 40%, and more desirably at least about 50%, of the polynucleotide (e.g., oligodeoxyribonucleotide, RNAi, etc.) is complexed with cationic lipids in the inner core of the liposomes. Typically, between about 60% and about 80% of the polynucleotide (e.g., oligodeoxyribonucleotide, RNAi, etc.) is complexed with cationic lipids in the inner core of the liposomes and between about 40% and about 20% of the polynucleotide (e.g., oligodeoxyribonucleotide, RNAi, etc.) is on the outer surface of the membrane. However, in some embodiments, more (e.g., about 90% or even about 95%) of the polynucleotide (e.g., oligodeoxyribonucleotide, RNAi, etc.) can be complexed with cationic lipids in the inner core of the liposomes. The high intraliposomal entrapment imparts sustained release of c-rafAON from the formulation.

[0067] Where the polynucleotide (e.g., RNAi or oligonucleotide) within the composition targets an oncogene, the invention provides a method of inhibiting the growth of neoplastic cells using such a composition. In accordance with the inventive method, the composition is

administered to such cells under conditions for the polynucleotide (e.g., RNAi or oligonucleotide) to enter the cells. Within the cells, the polynucleotide (e.g., RNAi or oligonucleotide) inhibits the activity of the oncogene to which it is targeted, which results in the inhibition of growth of such neoplastic cells. In this context, "inhibition" does not require a complete cessation of the growth or proliferation of such cells. It is sufficient for the method to retard neoplastic growth. However, it is desirable for the proliferation of the neoplastic cells to be substantially reduced or even eliminated.

[0068] When used against neoplastic cells, the method can be employed to cells *in vivo* or *in vitro*. For *in vitro* applications, the method can be used in research, e.g., to study neoplastic growth and proliferation. *In vitro* applications also can find use in developing therapies based on the inventive method. For such applications, the composition can be added to the culture medium of the cells or used in a similar manner as common lipid-based transfection agents (e.g., LIPOFECTIN®).

[0069] When used *in vivo*, the inventive method can be employed to treat a patient suffering from infectious diseases (e.g., bacterial infections, viral infections, etc.), auto-immune diseases (e.g., and cancer. Preferably, in such an embodiment, the composition comprising the cationic cardiolipin analogue and the polynucleotide (e.g., RNAi or oligonucleotide) targeting an oncogene is delivered to the patient under conditions sufficient for the polynucleotide (e.g., RNAi or oligonucleotide) to enter neoplastic cells in the patient. Within the cells, the polynucleotide (e.g., RNAi or oligonucleotide) inhibits the expression or activity of the oncogene, which results in an inhibition of the growth of the neoplastic cells within the patient. The cells can be, for example, cancer cells and can be within a tumor or metastasized.

[0070] As noted above, it is sufficient for the inventive method to retard the growth or proliferation of such cells. It is not a requirement that the inventive method completely eliminate neoplastic cells from the patient. Rather, it is sufficient for the inventive method to retard the growth or proliferation of the neoplastic cells within the patient. Preferably, the inventive method substantially inhibits the growth of the neoplastic cells, and, in some embodiments, the inventive method can result in tumor regression or elimination of neoplastic cells from the patient.

[0071] In accordance with the inventive method, the composition comprising the polynucleotide can be employed adjunctively with a second antineoplastic agent. Such a second antineoplastic agent can be, for example, a chemotherapeutic agent, a source of radiation, a gene therapy vector, or other antineoplastic agent. Specific examples of such chemotherapeutics include camptothecins (e.g., SN-38, irinotecan, etc.), doxorubicin, daunorubicin, methotrexate, adriamycin, tamoxifen, toremifene, cisplatin, epirubicin, docetaxal, paclitaxal, Gemzar, gemcitabine HCl, mixotrantrone, and other known

chemotherapeutics useful for treatment of cancer. When used adjunctively, the inventive composition comprising can be delivered prior to, concurrently with, or after the second antineoplastic agent. However employed, the inventive composition can enhance the efficacy of the second antineoplastic agent. Thus, the invention can be used to chemosensitize a tumor to the effect of chemotherapy. Similarly, the invention can be used to radiosensitize a tumor to the effects of radiation. In this respect, it is sufficient for the inventive method involving the use of a composition including the cationic cardiolipin analogue and the polynucleotide (e.g., RNAi or oligonucleotide) to contribute to the efficacy of the combined treatment regimen.

[0072] In another embodiment, the invention provides a method for validating a target for genetic inhibition. In accordance with this aspect of the invention, the inventive composition comprising a polynucleotide targeted to a particular gene is administered to cells expressing a target gene of interest under conditions for the polynucleotide (e.g., RNAi or oligonucleotide) to enter the cells. Within the cells, the polynucleotide (e.g., RNAi or oligonucleotide) inhibits the activity of the gene to which it is targeted. Thereafter, the effects of the inhibition of the gene are assayed.

[0073] The method of validating the target for genetic inhibition can be practiced using cells *in vitro* or *in vivo*, depending on the nature of the inquiry. The assay conducted to assess the effects of inhibition of the target gene can be qualitative — such as by probing for the presence or level of the targeted gene RNA or the expressed protein. In this sense, a reduction in the level of the targeted gene RNA or a reduction in the expression of the encoded protein of interest can serve to validate the target of the oligonucleotide, RNAi, or other inhibitor of gene expression. Alternative assays can be behavioral or phenotypic. For example, the composition can be administered to an animal or to a tissue within the animal, and the animal monitored to assess a behavioral or phenotypic change. In this sense, for example, genes mediating appetite can be validated by administering to an animal a composition comprising a genetic inhibitor of the putative appetite-regulating gene and observing the effect of the inhibition on the animal's appetite. A positive correlation between inhibition of the gene expression and a behavioral change in the animal can serve as a validation of the target. This method can rapidly identify and validate targets for therapeutic intervention. Traditionally, validation methods are costly and time-consuming, for example, requiring the generation of animal models, such as knock-out animals. The inventive method, in contrast, can much more rapidly and easily inhibit the expression of a putative target gene in a cell or an animal, which can accelerate and simplify the process of target validation.

[0074] Where the composition includes a fluorescent or luminescent cardiolipin analogues (or any other fluorescent or luminescent moiety), the invention provides a method



of tracking the migration of the composition within an animal, plant, or other desired system. In accordance with this aspect of the invention, a composition of the present invention including the fluorescent or luminescence moieties is introduced into the animal, plant, or other desired system. Immediately upon introduction or after a desired passage of time, the fluorescence or luminescence can be monitored by exposure to light, X-rays, or other ionizing radiation. This can be measured either through real-time observation or photographic detection. The method can be used to mark the migration of the lipid composition within the animal, plant, or other desired system. The method can serve to identify sites to which the lipid composition is concentrated, for example. This can serve to assess the effectiveness of a targeting moiety, if one is present in the composition. Additionally, the method can assist in determining the local concentration of the composition within an animal, plant, or other desired system. The local lipid concentration can also be detected by taking an organ tissue of interest or blood sample and treating with suitable chemical before the fluorescence or luminescence. Such information can be useful in designing and evaluating therapies wherein the local concentration of a therapeutic composition is of interest.

[0075] Of course, the method can be employed repeatedly. In this sense, after the initial detection of fluorescence, the moiety is again caused to fluoresce and the fluorescence again detected. This can serve to identify the change in position over time of the composition. Such information can be used to track the progression of tissues into which the composition may become concentrated, the migration of certain cell types. Alternatively, the method can help monitor the clearance of the composition from the animal, plant, or other desired system. Both fluorescence and luminescence methods can be useful depending on the application. Luminescence application may include studies requiring greater decay time such as effective monitoring of lateral diffusion of lipids across the membrane.

[0076] In another aspect, the invention entails the use of the compositions described herein as transfection agents. Thus, the composition can be admixed with a desired species or library of polynucleotide(s) and delivered to cells. The compositions of the present invention comprises desired polynucleotides, and the invention provides a method of transfecting cells with such polynucleotides by exposing the desired cell (or cells) to the composition under conditions sufficient for the polynucleotide to enter the cell(s) both *in vivo* or *in vitro*. For *in vitro* applications, the composition can be added to the culture medium of the cells or used in a similar fashion as common lipid-based transfection agents (e.g., LIPOFECTIN®). A desired quality of the present compositions, comprising cationic cardiolipin (especially PCL-2) is that they can serve as effective transfection agents even for primary cultures, whereas commonly employed transfection agents are generally ineffective in primary cultures. Moreover, the method of transfecting cells using the inventive

composition can be employed *in vivo*. As noted herein the method can be used to deliver polynucleotides that target specific genes, notably oncogenes. However, the method can be used to transfect cells *in vivo* with any desired polynucleotide. An advantage to employing the inventive compositions for this purpose is that the polynucleotide of interest can be delivered to cells without substantial toxicity, for example as is observed with LIPOFECTIN® and other lipid transfection agents. Thus, the invention provides a transfection agent that can be employed *in vivo* and *in vitro*, including primary and transformed cells.

[0077] Many aspects of the invention involve delivery of the composition to an animal, such as a veterinary patient or human. For delivery to a patient, the composition can be supplied in any suitable manner. For delivery to a tumor, for example, the composition can be formulated for injection directly into the tumor mass or infused through the circulation in the tumor. Alternatively, the composition can be delivered by injection (e.g., parenteral, intravenous, etc.) or adsorption (e.g., transdermal, transmucosal), as desired. Dermatological preparations can be applied suitable to skin or mucous tissues.

[0078] The following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope.

#### EXAMPLE 1

[0079] This example demonstrates the construction of nine PCL-2-based cationic liposomes for gene transfer. These formulations are suitable for mixing with any polynucleotide of choice for evaluating transfection efficiency.

[0080] Cationic cardiolipin analogue, PCL-2, and either DOPE or Cholesterol quantities corresponding each formulation (indicated in table 1A) were dissolved in chloroform in a round bottom flask. The solvent was removed under reduced pressure using a rotary evaporator to form a lipid film. The lipid film was further dried under vacuum overnight, and then hydrated with 20 mM HEPES buffer, under nitrogen, at 25 – 40 °C. The bulk cationic liposomes were extruded through 0.2 µm pore size polycarbonate filter three times, and 0.1 µm pore size polycarbonate filter five times. The extruded cationic liposomes were characterized by size and pH (Table 1A).

[0081] Cationic cardiolipin analogue, PCL-2, and either DOPE or Cholesterol quantities corresponding each formulation (indicated in Table 1B) were dissolved in chloroform in a round bottom flask. The solvent was removed under reduced pressure using a rotary evaporator to form a lipid film. The lipid film was further dried under vacuum overnight, and then hydrated with water, under nitrogen, at 25 – 40 °C. The bulk cationic liposomes were extruded through 0.2 µm pore size polycarbonate filter three times, and 0.1 µm pore size polycarbonate filter five times. The extruded cationic liposomes were characterized by size and pH (Table 1B).

## EXAMPLE 2

[0082] This example demonstrates the construction of a PCL-2-(CCLA)-based cationic liposome composition containing an antisense oligodeoxyribonucleotide

[0083] The composition is in the form of a lamellar complex having a percent molar ratio of 1,2 Dioleoyl-sn-glycero-3-phosphocholine (DOPC):cholesterol: and 1,3-Bis-(1,2-bistetradecyloxy-propyl-3-dimethylethoxyammonium bromide)-propane-2-ol (PCL-2), of 55:27:17 and having complexed therein c-rafAON (sequence sequence 5'-GTGCTCCATTGATGC-3') at total lipids:c-rafAON ratio of 90:1 and PCL-2:c-rafAON charge ratio of 2.2:1. This formulation also contains D-alpha tocopheryl acid succinate at 0.2 wt % and sucrose at 10-12 wt %.

[0084] c-rafAON (Lot ALH-01J-003-M) was obtained from Avecia Laboratories (MA, USA). DOPC, cholesterol, alpha tocopheryl acid succinate were obtained from Avanti Polar Lipids (Alabaster, AL, USA). PCL-2 was synthesized at NeoPharm Inc. Sucrose was obtained from Mallinckrodt (St. Louis, MO, USA).

[0085] To form this composition, the lamellar complex formulations were prepared by the thin film hydration method. The lipids (DOPC, PCL-2, cholesterol and D-alpha tocopheryl acid succinate) were dissolved in dehydrated alcohol. The lipid solution was evaporated to dryness using a rotary evaporator. After evaporation, the lipid residue was further dried overnight in a dessicator. Sucrose and c-raf AON were dissolved in de-ionized water. Then, the dried lipid residue was hydrated in the c-raf AON/sucrose solution to form a homogenous suspension. The size of the particles in the suspension was further reduced by extrusion through 0.8, 0.4, 0.2 and 0.1  $\mu$ m sized polycarbonate filters. The prototypes can also be prepared by reverse phase evaporation and ethanol injection methods.

[0086] The morphology of the formulation was determined by freeze-fracture microscopic method. Briefly, samples were quenched using sandwich technique and liquid nitrogen cooled propane at a cooling rate of 10,000 kelvin/second. The fracturing process was carried out in JEOL JED-9000 freeze-etching equipment and the exposed fracture planes were shadowed with platinum for 30 sec in an angle of 25-35 degree and with carbon for 35 sec (kV/60-70mA, 1/10-5 Torr). The platinum replicas were cleaned with concentrated, fuming HNO<sub>3</sub> for 24 to 36 hours, followed by repeating agitation with fresh chloroform/methanol (1:1 by volume) for at least five times. Subsequently, these cleaned replicas were examined with a JEOL 100 CX electron microscope.

[0087] The stability of the formulation was evaluated after storage at 2-8 °C and 25 °C for one month. The particle of the lamellar complex formulations was measured by dynamic light scattering technique (Nicom Particle Sizer). The drug entrapment efficiency of the formulation was determined by ultrafiltration and/or passage through Centricon filters with

100,000 molecular weight cut off. The free and entrapped drug and lipid contents were determined by HPLC methods.

[0088] The freeze-fracture replicas revealed complexes of unilamellar liposomes of mean size of about 50-200 nm. The c-rafAON formulation stored at 2-8 °C or 25 °C was stable for one month. The mean particle size, entrapment efficiency, c-rafAON and DOPC concentrations were not significantly different from initial values after 1 month storage at 2-8 °C and 25 °C. These results are presented in Table 2.

### EXAMPLE 3

[0089] This example demonstrates the construction of a PCL-2-based cationic liposome composition containing an antisense oligodeoxyribonucleotide.

[0090] The composition is a lamellar complex having a percent molar ratio of 1,2 Dioleoyl-sn-glycero-3-phosphocholine (DOPC):1cholesterol: and 3-Bis-(1,2-bistetradecyloxy-propyl-3-dimethylethoxyammonium bromide)-propane-2-ol (PCL-2, of 60:31:9 and having complexed therein c-rafAON at total lipids:c-rafAON ratio of 195:1 and PCL-2:c-rafAON charge ratio of 2.4:1. This formulation also contains D-alpha tocopheryl acid succinate at 0.2 wt % and sucrose at 10 wt %.

[0091] c-rafAON (Lot ALH-01J-003-M) was obtained from Avecia Laboratories (MA, USA). DOPC and cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL, USA) and alpha tocopheryl acid succinate from Sigma. PCL-2 was synthesized at NeoPharm Inc. Sucrose was obtained from Mallinckrodt (St. Lois, MO, USA).

[0092] The lamellar complex formulations were prepared by the thin film hydration method. The lipids (DOPC, PCL-2, cholesterol and D-Alpha Tocopheryl acid Succinate) were dissolved in dehydrated alcohol. Sucrose and c-raf AON were dissolved in de-ionized water. With constant stirring, the lipid solution was added to the c-rafAON solution to form multilamellar complexes. The size of the particles in the suspension was further reduced by extrusion through 0.8, 0.4, 0.2 and 0.1µm sized polycarbonate filters. The particle of the lamellar complex formulations was measured by dynamic light scattering technique (Nicomp Particle Sizer). The drug entrapment efficiency was determined by ultrafiltration and/or pass through Centricon filters with 100,000 molecular weight cut off, followed by the analysis of free c-rafAON in the filtrate by a HPLC method.

[0093] The release of c-rafAON from the lamellar complex formulation was determined by a reverse dialysis method. Briefly, 20 mL aliquot of the formulation was placed into 180 mL of stirred Phosphate Buffered Saline (PBS), pH 7.4 buffer, in which dialysis membrane tube (10,000 MW cutoff) containing 2 mL of the same PBS buffer was previously placed. At various time intervals, one dialysis tube is removed and analyzed for c-rafAON concentration.

[0094] The stability of the formulation was evaluated after storage at 2-8 °C for two months. The particle of the lamellar complex formulations was measured by dynamic light scattering technique (Nicomp Particle Sizer). The drug entrapment efficiency of the formulation was determined by ultrafiltration and/or passage through Centricon filters with 100,000 molecular weight cut off. The free and entrapped drug and lipid contents were determined by HPLC methods. It was observed that the lamellar complex formulation stored at 2-8 °C was stable for two months. The mean particle size, entrapment efficiency, c-rafAON and DOPC concentrations were not significantly different from initial values after 2 months of storage at 2-8°C. These results are presented in Tables 3A and 3B:

[0095] The physical and chemical stability of 8-fold diluted formulation samples in 5% Dextrose Solution was evaluated after storage at room and refrigerated temperatures (2-8 °C) for up to 48 hours. All stability samples were analyzed for appearance, pH, particle size, c-rafAON entrapment efficiency, c-rafAON concentration, DOPC concentration, cholesterol concentration and PCL2 concentration. The mean vesicle size was approximately 200 nm and entrapment efficiency of >99%. Less than 20 percent of c-rafAON was released from the formulation after 72 hours of dialysis. As presented in Table 3C, c-rafAON, DOPC, PCL-2, Cholesterol assay values, entrapment efficiency, pH and mean vesicle size were not significantly different from initial values after storage at 2-8°C or room temperature for 48 hours. The preparations were diluted in 5% Dextrose, USP.

#### EXAMPLE 4

[0096] This example demonstrates the *in vivo* efficacy and low toxicity of a composition according to the present invention containing a c-raf-1 antisense oligonucleotide as compared to other liposomal antisense oligonucleotide formulations.

#### Formulations

[0097] The test formulation (LErafAON-ETU) as prepared as described in Figure 1. Briefly, the lipid excipients, namely, DOPC, cholesterol, PCL-2 and D-Alpha tocopheryl acid succinate were dissolved in dehydrated ethanol and ii) the active ingredient a c-raf-1 antisense oligonucleotide (having the sequence 5'-GTGCTCCATTGATGC-3') and sucrose were dissolved in sterile water for injection. Ethanolic lipid solution was added to aqueous active solution to form liposomes. Following QS to batch weight, liposomes were extruded to meet size specifications and the ethanol was removed by rotary evaporation. After adjusting the batch weight to pre-solvent removal weight, the product was filtered through 0.22µ sterilizing filter and filled in sterile vials, stoppered and sealed.

[0098] The comparator formulation (DDAB-LErafAON formulation) contained the same concentration of antisense oligonucleotide but was formulated using commercially available cationic lipid, dimethyldioctadecyl ammonium bromide (DDAB).

[0099] The manufacturing process for non-sonicated 2-vial LerafaON formulation involved separate batch preparations of lyophilized lipids and rafaON. For lyophilized lipids, lipid excipients, namely, Egg Phosphatidylcholine (Egg PC), Cholesterol and DimethylDodecylAmmonium Bromide (DDAB) was first dissolved in tertiary butyl alcohol, the solution was filtered through 0.22  $\mu$ m sterilizing filter, filled into sterile vials and lyophilized. Lyophilized rafaON was manufactured by dissolving the drug substance in sterile water for injection, followed by its filtration through 0.22  $\mu$ m sterilizing filter, filling in sterile vials and lyophilization. Immediately prior to administration, the rafaON lyophilized vial is reconstituted with 0.9% Sodium Chloride, USP. The reconstituted rafaON is then transferred into a vial of lyophilized lipids, hydrated and sonicated for 10 minutes.

### **Pharmacology Studies**

[00100] The *in vivo* efficacy of the PCL-2 formulation, LerafaON-ETU, was compared to that of the DDAB formulation, LerafaON, in four studies.

#### **1. *In Vivo* Efficacy of LerafaON-ETU and LerafaON in SCID Mice Bearing Human Prostate (PC-3) Tumors**

[00101] The multiple dose therapeutic efficacies of the PCL-2 formulation LerafaON-ETU, and the control formulation, LerafaON, were evaluated in a SCID mouse xenograft model of human prostate cancer. PC-3 cells in the logarithmic growth phase were implanted subcutaneously (s.c.) in C.B.-17 SCID mice. Animals bearing tumors (50-125 mm<sup>3</sup>) were randomized into different treatment groups (5-7 mice/group), and mice received iv administration of control vehicle, LerafaON at 12.5 or 25 mg/kg/day, or LerafaON-ETU at 12.5 or 25 mg/kg/day, on Days 1, 2, 4, 5, 7, 8, 10, 11, 13 and 14. Efficacy was evaluated by comparing the % initial tumor volume (where Day 1 = 100%) of controls with LerafaON and LerafaON-ETU groups on Day 20.

[00102] Animals treated with 12.5 mg/kg/day of LerafaON or LerafaON-ETU did not show any tumor growth inhibition. However, animals treated with LerafaON or LerafaON-ETU at 25 mg/kg/day exhibited 15% and 44% tumor growth inhibition, respectively, compared to their respective controls (10% Sucrose). Both LerafaON and LerafaON-ETU were well tolerated, as no differences in body weight loss were observed.

[00103] The results of this study suggest that overall both the PCL-2 formulation (LerafaON-ETU) and the DDAB liposomal formulation (LerafaON) had comparable therapeutic efficacy against the human prostate tumor model in SCID mice.

## **2. *In Vivo* Efficacy of LErafAON-ETU and LErafAON in SCID Mice Bearing Human Breast (MDA-MB-231) Tumors**

[00104] The multiple dose therapeutic efficacies of the PCL-2 formulation, LErafAON-ETU and the control formulation, LErafAON, were evaluated in a SCID mouse xenograft model of human breast cancer. MDA-MB-231 cells in the logarithmic growth phase were implanted s.c. in C.B.-17 female SCID mice. Animals bearing tumors (50-100 mm<sup>3</sup>) were randomized into different treatment groups (8 mice/group), and received iv administration of control vehicle, or 25 mg/kg/day LErafAON or LErafAON-ETU on Days 1, 2, 4, 5, 7, 8, 10, 11, 13 and 14.

[00105] Maximal tumor growth inhibition was 65% on Day 12 and 59% on Day 15 for LErafAON and LErafAON-ETU, respectively, compared to the appropriate vehicle controls. No body weight loss was observed for any of the treatment groups.

[00106] The results suggest that LErafAON-ETU has equivalent therapeutic efficacy against the MDA-MB-231 human breast cancer tumor model as compared to the control LErafAON formulation.

## **3. *In Vivo* Efficacy of LErafAON-ETU and LErafAON and Their Combinations With Taxol® in SCID Mice Bearing Human Ovarian (SKOV-3) Tumors**

[00107] A multiple dose therapeutic efficacy study of LErafAON-ETU and LErafAON alone and in combination with Taxol® was conducted in a SCID mouse xenograft model of human ovarian cancer. SKOV-3 cells in the logarithmic growth phase were implanted s.c. in C.B.-17 SCID mice. Treatment with controls or test articles was initiated when tumor volume reached 50-100 mm<sup>3</sup>. Mice were randomized into treatment groups with 5-7 animals per group, then dosed intravenously with vehicle controls or with 25 mg/kg/day LErafAON or LErafAON-ETU on Days 1, 2, 4, 5, 7, 8, 10, 11, 12, and 13. Taxol® (25 mg/kg) was also administered intravenously to designated groups on Days 2, 5, and 9. Animals from combination therapy groups received Taxol® 2-3 hours after receiving the respective liposome formulation of rafAON.

[00108] LErafAON and LErafAON-ETU showed maximum tumor growth inhibition on Day 23 of 38% and 45%, respectively. Taxol® exhibited maximal tumor growth inhibition of 41% on Day 19. The combinations of Taxol® + LErafAON or Taxol® + LErafAON-ETU significantly regressed tumor growth by 90%. In addition, tumor growth to volumes greater than 400 mm<sup>3</sup> was delayed 13 days by Taxol® + LErafAON and 24 days by Taxol® + LErafAON-ETU.

[00109] The results of this study indicate that the PCL-2 formulation LErafAON-ETU has equivalent therapeutic efficacy as the control LErafAON against the SKOV-3 human ovarian cancer tumor model in SCID mice when administered as a single agent. However, in



combination treatment with Taxol®, the PCL-2 formulation, LEraFAON-ETU, resulted in superior tumor growth delay compared to the DDAB formulation (LEraFAON).

#### **4. *In Vivo* Efficacy of LEraFAON-ETU and LEraFAON and Their Combinations with Taxotere® in SCID Mice Bearing Human Prostate (PC-3) Tumors**

[00110] A multiple dose therapeutic efficacy study of the PCL-2 formulation, LEraFAON-ETU, and the control DDAB formulation, LEraFAON, alone and in combination with sub-therapeutic doses of Taxotere® was conducted in a SCID mouse xenograft model of human prostate cancer. PC-3 cells in the logarithmic growth phase were implanted s.c. in C.B.-17 SCID mice. Animals bearing tumors (50-100 mm<sup>3</sup>) were randomized into different treatment groups (5-7 mice/group), and controls or test articles (25 mg/kg/day LEraFAON or LEraFAON-ETU) were administered intravenously for 5 consecutive days. Taxotere® was dosed to designated groups at 10 mg/kg on Day 2 and 5 mg/kg on Day 5. Efficacy was evaluated by comparing the % initial tumor volume (where Day 1 = 100%) of controls with treated groups on Day 21.

[00111] Tumor growth was inhibited 7% and 40% for mice dosed with LEraFAON and LEraFAON-ETU, respectively, compared to their controls. Taxotere® alone resulted in 83% tumor growth inhibition, whereas, in combination treatment, LEraFAON + Taxotere® led to a similar tumor growth inhibition of 81%. Animals dosed with the PCL-2 formulation, LEraFAON-ETU, + Taxotere® showed 99% tumor growth inhibition on Day 21. These results are presented in Figures 4A and 4B.

[00112] The results of this study suggest that the PCL-2 formulation, LEraFAON-ETU, in combination with Taxotere® has greater therapeutic efficacy in comparison to the control DDAB formulation LEraFAON + Taxotere® combination.

#### **Pharmacokinetics**

[00113] The pharmacokinetics of the PCL-2 rafaON formulation, LEraFAON-ETU, was compared to that of the control (DDAB) liposome formulation, LEraFAON, in CD2F1 mice.

##### **1. Bioanalytical Methods**

[00114] Bioanalytical methods were developed to quantify total rafaON in mouse plasma and mouse tissues containing LEraFAON or LEraFAON-ETU, and were used to quantify rafaON in plasma and tissue samples from the single dose pharmacokinetics and tissue distribution study in mice. RafaON was extracted from mouse plasma samples by solid phase extraction (SPE), then quantified using LC-MS/MS. The method was found to be linear over the range 25-5000 ng/mL in mouse plasma. Tissues were homogenized mechanically to form a 5% (w/v) homogenate; this was then subjected to protein precipitation



for sample preparation. Total rafaON was then quantified in samples by an LC-MS/MS method with linearity from 50-10,000 ng/mL tissue homogenate.

## **2. Pharmacokinetics and Tissue Distribution Study of LEraFAON-ETU and LEraFAON in Mice**

[00115] A single dose pharmacokinetics and tissue distribution study of the control LEraFAON and the PCL-2 formulation (LEraFAON-ETU) was conducted following a 30 mg/kg intravenous dose to male CD2F1 mice. Three mice/group/time points were sacrificed at 5, 15 and 30 minutes and at 1, 2, 4, 8, 24, and 48 hours after formulation administration. Blood samples were processed for plasma, and plasma and tissues were assayed for rafaON concentrations.

[00116] The plasma  $C_{max}$  for animals dosed with LEraFAON-ETU was approximately twice that for animals dosed with LEraFAON. Total exposure (AUC<sub>0-48h</sub> and AUC<sub>0-∞</sub>) was 2.3-fold higher for LEraFAON-ETU than for LEraFAON dosed mice. Clearance of LEraFAON was 2.5-fold faster and volume of distribution ( $V_z$ ) was almost 4-fold greater than for LEraFAON-ETU.

[00117] RafaON was rapidly distributed to the tissues following dose administration. The order of increasing tissue exposure over 48 hours was lungs > spleen > liver > kidneys > heart, for both formulations. RafaON concentrations were found to be greater in mice dosed with the PCL-2 formulation (LEraFAON-ETU) in comparison to the DDAB formulation (LEraFAON).

## **Toxicology**

[00118] The toxicity of the PCL-2 rafaON formulation, LEraFAON-ETU, was compared to that of the control liposome formulation, LEraFAON, in CD2F1 mice.

### **Multiple Dose Toxicity in Mice**

[00119] A comparative multiple dose toxicity study of the control (LEraFAON) and the PCL-2 formulation (LEraFAON-ETU) was conducted in male and female CD2F1 mice. Animals were randomized into treatment groups (10/sex/group) and were dosed intravenously with 35 mg/kg/day LEraFAON or LEraFAON-ETU for 5 consecutive days.

[00120] No significant body weight loss or clinical signs of toxicity were observed in mice dosed with the PCL-2 formulation (LEraFAON-ETU). The mortality of animals dosed with LEraFAON-ETU was 0% for both males and females, and was 100% for males and 40% for females dosed with LEraFAON. All males and 40% of the females dosed with LEraFAON were found dead by Day 5, and all showed clinical signs of toxicity (hunched posture, rough coats, and dehydration). No statistically significant body weight loss was observed in the males, however females had 12% weight loss on Day 8, but recovered by Day 17. The

females that survived dosing with LErafAON beyond Day 5 were lethargic, but recovered by Day 9. The results of this study are presented in Figure 4C.

[00121] Histopathological evaluations concluded that the mortality of mice dosed with LErafAON was caused by pulmonary hemorrhage. Lesions were observed in the spleen, lungs, and liver of mice dosed with LErafAON, but not in mice dosed with the PCL-2 formulation (LErafAON-ETU). Clinical pathology tests showed no significant differences in clinical chemistry between mice dosed with controls and those dosed with LErafAON or LErafAON-ETU.

[00122] The results of this study indicate that the PCL-2 formulation (LErafAON-ETU) is less toxic to CD2F1 mice than the control formulation LErafAON.

#### EXAMPLE 5

[00123] This example demonstrates the transfection efficiency of the inventive composition in transfecting cells. The example highlights the ability of the PCL-2 formulation to transfect a variety of cell lines including primary cells, and the results reveal that the inventive composition can mediate transfection of cells more efficiently in cell lines tested than commonly employed transfection reagents, such as LIPOFECTIN® and Lipofectamine 2000™.

[00124] The composition for use in the experiments reported in this example (referred to herein as "NeoPhectin™") was prepared using procedure described in Example 1. The inventive formulation contained PCL-2 and DOPE.

##### 1. Transfection Efficiency in CHO Cells

[00125] NeoPhectin™ was evaluated for transfection in Chinese hamster ovary (CHO) cells using CMV-luciferase reporter gene plasmid and compared with LIPOFECTIN®. One µg DNA was added to CHO cells in the presence and absence of NeoPhectin™ or LIPOFECTIN® in a 24 well plate. Cells were incubated for 4h in serum-free medium, then changed to medium containing 10% serum for further 24h. Protein concentration was measured by the bicinchoninic acid (BCA) protein assay using bovine serum albumin as a standard, and transfection efficiency was determined by measuring relative light units/mg of cell protein. The results of this experiment (see Figure 5A) indicate that at all concentrations NeoPhectin™ exhibited significantly greater transfection efficiency than LIPOFECTIN®.

##### 2. Transfection Efficiency in COS-1 Cells

[0100] NeoPhectin™ was evaluated for transfection in COS-1 cells using LacZ plasmid. Four µg of plasmid pcDNA3.1/His/LacZ was incubated with NeoPhectin™ or LIPOFECTIN® in serum free media at room temperature for 30 min. Subsequently, reagent mixture was added to 0.3 million cells per well and incubated for 6 h in serum free medium.

The mixture was replaced with 10% serum media and further incubated for 48h. The transfection efficiency was measured by staining with  $\beta$ -Gal staining kit and counting number of cells transfected per field using inverted microscope. The results of this experiment (see Figure 5B) indicate that at all concentrations, NeoPhectin™ exhibited significantly greater transfection efficiency than LIPOFECTIN®.

### **3. NeoPhectin™ Mediated Delivery of RNAi in Primary Rat Lung Microvessel Endothelial Cells (RLMVEC)**

[0101] NeoPhectin™ was evaluated for delivery of RNAi in primary rat lung microvessel endothelial cells (RLMVEC). RNAi was admixed with NeoPhectin™ and various concentrations were exposed to RLMVEC (see Figure 5C). The efficiency of transfection was assessed by measuring the uptake of the RNAi oligos in relative units. The results of this experiment (see Figure 5C) indicate that NeoPhectin™ efficiently delivered RNAi to the RLMVEC.

### **4. Transfection Efficiency of NeoPhectin™ in BALB/3T3 Cells**

[0102] The transfection efficiency of NeoPhectin™ was evaluated using  $\beta$ -galactosidase reporter gene assay. After cultured in 96-well plates overnight, BALB/3T3 cells were transfected with 0.2  $\mu$ g/well of pSV- $\beta$ -galactosidase vector and 0.375 to 6  $\mu$ g/well of NeoPhectin™ or Lipofectamine 2000™ in serum-free medium for 6 hours. After transfection, the liposome-DNA complex was replaced with serum-containing medium. The  $\beta$ -galactosidase activity was determined 24 hr after transfection and plotted against exogenous  $\beta$ -galactosidase standards. The results of this study (see Figure 5D) reveal that NeoPhectin™ exhibited significantly greater transfection efficiency than Lipofectamine 2000™.

### **5. Inhibition of a Cytokine Receptor (TbRII) Expression with dsRNA Interference using NeoPhectin™ in Rat Pulmonary Microvessel Endothelial Primary Cell Culture (PLMVEC)**

[0103] Rat Pulmonary Microvessel Endothelial Cells (PLMVEC) primary cell culture was used for RNAi to inhibit the production of a cytokine receptor involved in tumor formation. NeoPhectin™ was used to transfect PLMVEC cells using specific double stranded RNA (dsRNA). Cells were exposed to NeoPhectin™ and oligonucleotides at different times at 37°C in 5% CO<sub>2</sub>. Inhibition was observed within 48 hours and inhibition increased at 72 and 96 hours (see Figure 5E)

## **6. Effect of NeoPhectin™ Mediated Delivery of RNAi (200nM) Oligonucleotides in Primary Cells After 48 Hours**

[0104] NeoPhectin™ was evaluated for delivery of RNAi in RLMVEC primary cells. The TbRII expression was detected using indirect immunofluorescence. Primary antibody was Rabbit anti-TbRII, and secondary antibody was Goat antiRabbit conjugated with FITC. RNAi was admixed with NeoPhectin™ and 200 nM was exposed to primary cells (see Figure 5C). The effect of NeoPhectin™ mediated delivery of the RNAi was measured by fluorescence. After 48 hours, the NeoPhectin™ treated cells exhibited markedly reduced fluorescence as compared to control cells (see Figure 5F), indicating the inhibition of the gene mediating the fluorescence within the cells. These results indicate that NeoPhectin™ can efficiently deliver RNAi to primary cells and that the RNAi can function within the cells to inhibit gene expression.

## **7. NeoPhectin™ (2.5 µg/mL) Mediated Delivery of RNAi Oligonucleotides Inhibits T<sub>RII</sub> Gene Expression**

[0105] NeoPhectin™ was evaluated for delivery of RNAi in Primary Rat Lung Microvessel Endothelial Cells (RLMVEC), Primary Human Umbilical Vein Endothelial Cells (HUVEC), and Primary Human Pulmonary Artery Endothelial Cells (HPAEC). RNAi targeted to the T<sub>RII</sub> gene was admixed with NeoPhectin™ and 2.5 µg/mL were exposed to the three cell types (see Figures 5G, 5H, and 5I). The efficiency of transfection was assessed by measuring the percent gene expression relative to control cells. The results of this experiment (see Figure 5G, 5H, and 5I) indicate that NeoPhectin™ efficiently delivered RNAi to the cells.

## **EXAMPLE 6**

[0106] This example demonstrates that the compositions of the present invention represent a novel cationic cardiolipin platform for safe and enhanced *in vitro* and *in vivo* delivery.

### **A. Materials and Methods**

#### **1. Preparation of NeoPhectin-AT™ (PCL-2-(CCLA)-Based Liposomes)**

[0107] Cholesterol and DOPE (dioleoylphosphatidylethanolamine) used as helper lipids were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Several CCLA-based liposomes were prepared from CCLA and helper lipids at different molar ratio using thin film hydration method. In brief, CCLA, DOPE or cholesterol were dissolved in chloroform in a round bottom flask. The solvent was removed under reduced pressure using a rotatory evaporator to form a lipid film, and further dried under vacuum overnight. The lipid film was hydrated with 10% sucrose in RNase-free water under nitrogen, at 25-40°C. RNase-free water

was obtained from Sigma (St. Louis, MO). The bulk cationic liposomes were extruded through 0.2  $\mu\text{m}$  pore size polycarbonate filter three times and 0.1  $\mu\text{m}$  pore size polycarbonate filter five times. The extruded liposomes were sterilized by filtering through 0.22  $\mu\text{m}$  sterile filter unit (Millipak® 20 positively charged). The prepared liposomes (NeoPhectin-AT™) had a mean particle size of 110-120 nm. The size of the liposomes was characterized using a light scattering particle sizer (Nicomp Model 380, Santa Barbara, CA).

## 2. Preparation of Cell Culture

[0108] A549 (human lung cancer), PC-3 (human prostate cancer), SK-OV-3 (human ovarian cancer), MX-1 and MDA-MB-231 (human breast cancer) cells were obtained from the National Cancer Institute (Frederick, MD) and maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (HI-FBS). CHO (Chinese Hamster Ovary), BALB/3T3 (murine embryonic fibroblasts) and HepG<sub>2</sub> (human hepatocellular carcinoma) were purchased from American Type Culture Collection (Manassas, VA). CHO cells were maintained in Kaighn's modification of Ham's F12 medium (F12K) with 10% HI-FBS. BALB/3T3 cells were maintained in Dulbecco's Modified Eagle's Medium with 10% calf serum. HepG<sub>2</sub> cells were maintained in Eagle's Minimum Essential Medium with 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids and 10% HI-FBS. All culture medium and reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA).

## B. Liposome Studies

### 1. *In vitro* efficiency

[0109] One day prior to transfection, CHO (25 000 cells/well), BALB/3T3 (25 000 cells/well), MX-1 (50 000 cells/well), and A549 (25 000 cells/well) were seeded in 96-well plates and HepG<sub>2</sub> (200 000 cells/well) in 24-well plates, and cultured overnight in a 5% CO<sub>2</sub> incubator. The cells were then washed with phosphate-buffered saline to remove the residual serum before adding the transfection mixture.

[0110] The transfection efficiency of PCL-2-(CCLA)-based liposomes was determined using  $\beta$ -galactosidase ( $\beta$ -gal) reporter gene assay for CHO, BALB/3T3 and HepG<sub>2</sub> cells and luciferase reporter gene assay for MX-1 and A549 cells. The pSV- $\beta$ -galactosidase control vectors were purchased from Promega (Madison, WI) and the gWIZ luciferase vectors were purchased from Gene Therapy Systems (San Diego, CA). Total amount of DNA plasmids was 0.2  $\mu\text{g}$ /well in 96-well plates and 1  $\mu\text{g}$ /well in 24-well plates. The plasmids were diluted with OptiMEM I Reduced Serum Medium (Invitrogen, Carlsbad, CA) in cell culture tubes. Equal volume of OptiMEM I medium was used to dilute CCLA-based liposome. Appropriate amount of CCLA-based liposome was resuspended in OptiMEM I medium to obtain CCLA/DNA (+/-) charge ratio of 8:1 and diluted in series to 4:1, 2:1, 1:1 and 1:2 with OptiMEM I medium and incubated at room temperature for 5 minutes. DNA was then added

drop by drop to CCLA-based liposome dilution and incubated at room temperature for additional 30 minutes. After incubation, the DNA/CCLA-based liposome complex was added to the cells and incubated further for 4-6 hours. After transfection, the residual DNA/CCLA-based liposome complex was washed off and the cells were replenished with regular culture medium and incubated up to 24 hours.

[0111]  $\beta$ -gal reporter gene assay was performed using  $\beta$ -gal Reporter Gene Assay System (Promega, Madison, WI) according to the manufacturer's protocol. The optical density (OD) of the sample was read at 414 nm using a plate reader (Thermo Electron, Franklin, MA). The amount of  $\beta$ -gal expression was calculated based on the exogenous  $\beta$ -gal standard. The expression of luciferase was measured using Luciferase Assay System (Promega, Madison, WI). The assay reagent was mixed with 20  $\mu$ l of cell lysate and the relative light unit (RLU) was determined by a luminometer (Thermo Electron, Franklin, MA).

[0112] To determine the optimal combination for the CCLA-based liposome transfection, two most commonly used helper lipids, cholesterol and DOPE, were formulated with CCLA at different molar ratio and screened for their transfection efficiency *in vitro* using  $\beta$ -gal reporter gene assay. The evaluation results showed that the formulation composed of CCLA: DOPE at molar ratio of 1:2 had high transfection among the cell lines tested.

[0113] In CHO, BALB/3T3, A549 and MX-1 cells, the peak gene expression from reporter genes was obtained at 2:1 of CCLA/DNA (+/-) charge ratio, whereas in HepG<sub>2</sub> cells, the peak activity was at charge ratio of 1:1 (Fig. 6A).

## 2. *In vivo* efficacy

[0114] The transfection efficiency of PCL-2-(CCLA)-based liposome in male Balb/c mice was determined using Luciferase reporter gene assay. The gWIZ luciferase vectors (50  $\mu$ g/mouse) were gently mixed to the surface of CCLA-based liposome. The DNA/CCLA-based liposome complex was injected to mice intravenously through tail vein within 8 hours of the preparation. Balb/c mice were obtained from Charles River Laboratories (Portage, MI). Equal quantity of DNA was delivered with *In Vivo* GeneSHUTTLE™, a DOTAP-based formulation (QBiogene, Carlsbad, CA) following the manufacturer's recommended protocol. The animals were sacrificed 24 hours after the injection. The lung and heart tissues were quickly removed and frozen in an ethanol/dry ice bath. The tissues were then homogenized in Cell Culture Lysis Reagent (Promega, Madison, WI) using an Autogizer (Tomtec, Hamden, CT). The homogenate was centrifuged at 12 000 rpm (16 000g) for 10 minutes at 4°C and the supernatant was transferred to a separate tube. A 20  $\mu$ L aliquot from the supernatant was used to measure the luciferase activity. The protein concentration of each tissue sample was determined using RC DC protein assay according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA).

[0115] Several formulations composed of CCLA and cholesterol or DOPE at different molar ratios, were screened. Interestingly, the molar ratio of CCLA: DOPE at 1:2, the same composition to the *in vitro* formulation, showed better transfection activity than CCLA with cholesterol. When different charge ratios of CCLA/DNA (+/-) were tested with 50 µg of luciferase reporter gene, the maximum expression of luciferase was observed at 5:1 (+/-) both in lung and heart tissues (Fig.6B). In contrast, no expression was observed in the mice treated with DNA or CCLA-based liposome alone.

[0116] The transfection efficiency of CCLA-based liposomes was also compared with another commercially available cationic liposome (*In Vivo* GeneSHUTTLE™) in male Balb/c mice. The DOTAP-based *In Vivo* GeneSHUTTLE™ is one of very few commercially available liposome used for *in vivo* experiments. The luciferase reporter gene (50 µg/mouse) was delivered with either the optimal charge ratio (+/- 5:1) of CCLA-based liposomes or the *In Vivo* GeneSHUTTLE™ using the manufacturer's recommended condition. Our results showed that the luciferase gene expression in lungs, if delivered by CCLA-based liposome, was approximately 7 fold higher than by *In Vivo* GeneSHUTTLE™ (Fig. 6C). The calculated charge ratio of DOTAP to DNA was also approximately 5:1.

### 3. *In vivo* toxicity

[0117] The toxicity of PCL-2-(CCLA)-based liposome was determined in male Balb/c mice. The single-dose toxicity of the CCLA-based liposome was evaluated by injecting mice with 100 mg/kg of CCLA-based liposome via the tail vein. The DOTAP-based *In Vivo* GeneSHUTTLE™ was included for comparative analysis. The multiple-dose toxicity was also examined by injecting mice with 50, 75 or 100 mg/kg of the CCLA-based liposome via the tail vein for 3 consecutive days. Three animals were used in each group. The mortality, body weight and clinical signs of toxicity were monitored daily for 14 days.

[0118] No mortality was observed in CCLA liposome-treated group whereas 66.6% mortality was recorded for the *In Vivo* GeneSHUTTLE™-treated group (Table 6). In the multiple-dose toxicity study, mice were injected with 50, 75 or 100 mg/kg of CCLA-based liposome for 3 consecutive days. No mortality was seen in the groups injected with 50, and 75 mg/kg/day, whereas 33.3% mice died 3 days after receiving 100 mg/kg/day of CCLA-based liposome (Table 6). There was no significant body weight loss in 50 and 75 mg/kg treated groups and only up to 10.1% weight loss occurred in mice treated with 100 mg/kg/day of CCLA-based liposome. The surviving animals completely recovered 10 days after the initial treatment. Our results indicated that the toxicity of CCLA-based liposome was significantly lower than *In Vivo* GeneSHUTTLE™.

## C. Delivery of siRNA

### 1 *In vitro* efficacy

[0119] The therapeutic efficacy study of siRNA against c-raf was tested by transfecting c-raf siRNA to A549, PC-3, MDA-MB-231 and SK-OV-3 cancer cells with PCL-2-(CCLA)-based liposome. siRNA duplex was designed to target the specific sequence of human mRNA for c-Raf gene (accession number X03484). The selected sequence was screened in BLAST search to verify that only c-Raf mRNA was targeted. Synthetic siRNA duplex was custom synthesized at Dharmacon (Lafayette, CO) in the 2'-deprotected, desalted form. The sense and antisense sequence of the duplex were AUUCCUGCUCAAUGGAUUUdTdT and AAUCCAUUGAGCAGGAAUdTdT, respectively. The mismatch double stranded siRNA sequence (5'-AGCUUGCCAUCCAUGCUAUdTdT-3' and 5-AUAGCAUGGAUGGCAAGCUDdTdT-3') was also obtained from Dharmacon (Lafayette, CO). The mismatch sequence was also screened in BLAST search to verify that there was no homology with c-raf mRNA sequence.

[0120] A549, PC-3, MDA-MB-231 and SK-OV-3 cells were transfected with siRNA alone, or CCLA-based liposome alone, or the combination of both. Approximately 10 000 cells per well were plated in a 96 well plate and transfected with 400 nM of c-raf siRNA or/and 10 µg/mL CCLA-based liposome for 6 hours. After transfection, the c-raf siRNA/CCLA-based liposome complex was washed off and the cells were replenished with fresh medium and incubated for 72 hours. The cytotoxicity was measured 72 hours after the transfection using a sulforhodamine B (SRB) assay. The results showed that 400 nM of siRNA, delivered by CCLA-based liposome, induced 54%, 62%, 33% and 34% cytotoxicity in A549, PC-3, MDA-MB-231 and SK-OV-3 cells, respectively, as compared to the control cells. No apparent cytotoxicity was seen in cells treated with free siRNA or liposome alone (Fig. 6D).

### 2. *In vivo* efficacy

[0121] The therapeutic efficacy of siRNA against c-raf was further tested against human breast tumor xenograft model in SCID mice. MDA-MB-231 cells ( $2 \times 10^6$  cells in 0.1 ml HBSS) were injected subcutaneously to female C.B-17 SCID mice (4-5 weeks old from Harlan Sprague Dawley, Indianapolis, IN). When tumors reached volume of 50-100 mm<sup>3</sup>, mice were randomized into four groups. Three groups were treated with free siRNA in RNase-free and DNase-free water, mismatched c-raf siRNA/PCL-2-(CCLA)-based liposome, or c-raf siRNA/CCLA-based liposome, respectively. The injection was conducted twice (b.i.d) per day for 5 days through tail vein injection at 7.5 mg/kg/day of siRNA. Sucrose (10%, w/v) at equal volume of liposome complex was injected in the fourth group as control. The tumor size was measured twice per week with a caliper, and the tumor volume was



calculated using following formula:  $a \times (b/2)^2 \times \pi$ , where  $\pi = 3.14$ ,  $a$  and  $b$  are the tumor length and width, respectively. Mice were sacrificed 15 days after the initial treatment when the tumors of the control group had reached the maximum volume ( $\sim 400 \text{ mm}^3$ ). All data were expressed as mean  $\pm$  S.E.M. The comparison between groups was conducted with Student's  $t$  test (two tailed, unpaired). It was considered to be statistically significant if  $p$  value  $< 0.05$ .

[0122] The treatment of c-raf siRNA/CCLA-based liposome complex exhibited 73% tumor growth inhibition as compared to free c-raf siRNA group 8 days after the initial treatment (Fig. 6E). The difference was found to be statistically significant ( $p < 0.05$  in  $t$  test). In contrast, 10% sucrose, free c-raf siRNA or mismatch siRNA delivered by CCLA-based liposome had no significant effect on the tumor growth.

#### EXAMPLE 7

[0123] This example demonstrates that the compositions of the present invention comprising DOPE, cholesterol, cardiolipin and rafsiRNA are able to (1) effectively knock down the expression of raf-1 and cyclin D1 in tumor tissues or PC-3 tumor bearing SCID mice and (2) enhance the therapeutic index of Taxotere when administered in combination.

##### A. Materials and Methods

###### 1. RAFsiRNA design and synthesis

[0124] The rafsiRNA sequence was designed from within the coding region of *c-raf-1* mRNA (nt 600-618, GenBank accession number X03484) and screened in the BLAST search to verify a significant sequence homology with *c-raf-1* mRNA. Proprietary synthetic double stranded siRNA against Raf-1 was obtained from Dharmacon (Lafayette, CO).

###### 2. Preparation of Liposomes

[0125] PCL-2-(CCLA)-based liposomes (NeoPhectin-AT<sup>TM</sup>) were prepared as described in Example 6 from PCL-2 and helper lipids using a thin film hydration method.

###### 3. Cell Culture

[0126] Human prostate cancer cells (PC-3) were obtained from the Biological Testing Branch, Developmental Therapeutics Program of National Cancer Institute (Frederick, MD). Tumor cells were propagated in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/mL of penicillin, and 100  $\mu\text{g}/\text{mL}$  of streptomycin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Culture medium and related reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA).

###### 4. Formulation of rafsiRNA-Neophectin complex

[0127] Lyophilized rafsiRNA was hydrated with nuclease-free water. This siRNA solution was slowly dispensed into a tube containing an appropriate volume of

NeoPhectin-AT<sup>TM</sup>, mixed gently, and kept at room temperature for 15-20 min before administration to mice. The mismatch siRNA-NeoPhectin complex was prepared as described above.

## **5. Animals and Tumor transplantation**

[0128] Male CB-17 SCID mice (3-4 weeks old) were obtained from Harlan Sprague Dawley Laboratories (Indianapolis, IN). Mice were handled aseptically and housed in microisolators (Tecniplast, Italy). Animals were fed with autoclaved TEKLAD 18% protein rodent diet (Harlan Teklad, Madison, WI) and autoclaved water ad libitum. Logarithmically growing PC-3 cells were injected subcutaneously (5x10<sup>6</sup> cells/0.1ml/animal). Tumors were allowed to grow to approximately 120 mm<sup>3</sup>.

## **6. Western-blot analysis**

[0129] Adslfj PC-3 tumor bearing SCID mice were randomized into the following treatment groups (treatment was administered once daily, QD or twice daily, BID): mismatch siRNA-NeoPhectin (7.5 mg/kg, BID, x 5), free rafsiRNA (7.5 mg/kg, BID, x 5), rafsiRNA-NeoPhectin (7.5 mg/kg, QD or BID, x 5), Taxotere<sup>®</sup>, free rafsiRNA (7.5 mg/kg, QD, x 5) + Taxotere<sup>®</sup>, rafsiRNA-NeoPhectin (7.5 mg/kg, QD, x 5) + Taxotere<sup>®</sup>, and control (normal saline, BID x 5). In the Taxotere<sup>®</sup> alone treatment group and in the combination treatment groups, Taxotere<sup>®</sup> was administered on Day 2 (10 mg/kg) and on Day 5 (5 mg/kg). All treatments were given intravenously via tail vein. Tumor tissues were excised within 6-8 h after the last treatment. Raf-1 and cyclin D1 protein expression were examined in tumor tissue homogenates by immunoblotting using respective antibodies (Santa Cruz Biotech, Santa Cruz, CA). The blots were reprobbed with polyclonal anti-GAPDH antibody (Trevigen Inc., Gaithersburg, MD). Raf-1 and cyclin D1 protein levels were quantified and normalized against GAPDH expression in the corresponding lanes using ImageQuant software (Amersham Pharmacia Biotech, Piscataway, NJ).

## **7. Antitumor efficacy**

[0130] Therapeutic efficacies of rafsiRNA-NeoPhectin and a combination of rafsiRNA-NeoPhectin and Taxotere<sup>®</sup> were evaluated in human prostate tumor model (PC-3). Animals were treated intravenously via tail vein with free rafsiRNA, rafsiRNA-NeoPhectin, Taxotere<sup>®</sup>, free rafsiRNA + Taxotere<sup>®</sup>, or rafsiRNA-NeoPhectin + Taxotere<sup>®</sup>. The doses and schedules of treatment were the same as described in the western blot analysis. Control groups received i.v. doses of normal saline on the same dosing schedule as for rafsiRNA-NeoPhectin. Tumor sizes were monitored once or twice weekly by caliper measurements of length and width, and the tumor volumes were calculated using the formula:

length  $\times$  (width/2)<sup>2</sup>  $\times$   $\pi$ . The mean tumor volume  $\pm$  SEM for each treatment group was calculated. The statistical significance between the groups was determined by Student's *t* test.

**B. Systemic administration of rafsiRNA-NeoPhectin inhibits Raf-1 expression in PC-3 tumor tissues**

[0131] Administration of free rafsiRNA at 7.5 mg/kg, BID for five consecutive days resulted in 63% expression of Raf-1 in PC-3 tumor tissues as compared to the control normal saline group (100%) (n = 3). Animals treated with rafsiRNA-NeoPhectin at QD or BID schedule (7.5 mg/kg for 5 consecutive days) showed 21% and 11% Raf-1 expression, respectively (Figure 7A and 7B) (n = 2/3).

[0132] Treatment with a mismatch siRNA-NeoPhectin at 7.5 mg/kg  $\times$  5, BID for five consecutive days had no inhibitory effect on Raf-1 level (n = 3). Treatment with Taxotere<sup>®</sup> alone or a combination of free rafsiRNA and Taxotere treatments showed 43% and 51% Raf-1 expression, respectively (n = 3).

[0133] Remarkably, animals treated with a combination of rafsiRNA-NeoPhectin and Taxotere<sup>®</sup> showed only 2% Raf-1 expression remaining in the PC-3 tumor tissues as compared to control group (n = 3) (Figure 7C and 7D).

**C. RafsiRNA-NeoPhectin-mediated inhibition of Raf-1 expression is associated with decreased expression of PC-3 tumor tissues.**

[0134] Cyclin D1 is an important regulator of cell cycle progression. In addition, cyclin D1 functions as an oncogene in several tumor types. We evaluated the effect of rafsiRNA-NeoPhectin treatment on cyclin D1 expression in tumor tissues. No significant difference in cyclin D1 expression in PC-3 tumor tissue was observed in animals treated with either free rafsiRNA (7.5 mg/kg, BID,  $\times$ 5) or mismatch siRNA-NeoPhectin (7.5 mg/kg, BID,  $\times$ 5) (n=3) (Figure 7E and 7F).

[0135] Treatment of mice with rafsiRNA-NeoPhectin at 7.5 mg/kg (QD,  $\times$ 5) decreased cyclin D1 expression by 58% as compared to normal saline control (100%) (n = 3). Moreover, treatment with rafsiRNA-NeoPhectin at 7.5 mg/kg (BID,  $\times$ 5) caused a more pronounced repression of cyclin D1 expression (75%). Taxotere<sup>®</sup> alone or a combination of Taxotere<sup>®</sup> and free rafsiRNA did not inhibit cyclin D1 expression in tumor tissues. Consistently, a combination of rafsiRNA-NeoPhectin (7.5 mg/kg, QD,  $\times$ 5) and Taxotere<sup>®</sup> treatments led to inhibition of cyclin D1 expression comparable to siRNA-NeoPhectin treatment alone (rafsiRNA-NeoPhectin + Taxotere, 65%, siRNA-NeoPhectin, 75%; n = 3) (Figure 7G and 7H).

**D. RafsiRNA-NeoPhectin inhibits tumor growth and improved response to Taxotere in PC-3 tumor xenograft model.**

[0136] Free rafsiRNA at 7.5 mg/kg (BID, x5) had no effect on tumor growth. Animals treated with Taxotere® alone or Taxotere in combination with free rafsiRNA showed 71% and 66% tumor growth inhibition, respectively, compared to control group animals ( $p < 0.05$ , day 18 post-treatment initiation). Animals treated with rafsiRNA-NeoPhectin 7.5 mg/kg QD x5 or 7.5 mg/kg BID x5 showed inhibition of tumor growth by 39% and 49%, respectively as compared to the control group ( $p < 0.05$  day 18 post-treatment initiation).

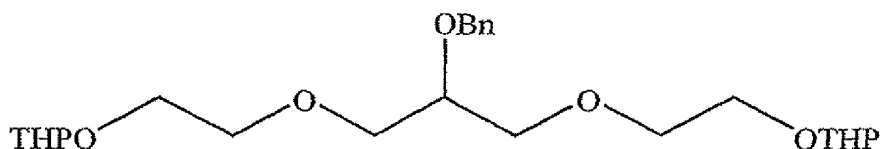
[0137] Most noteworthy was the effect of a combination of rafsiRNA-NeoPhectin and Taxotere on tumor growth. On day 18 after treatment initiation, a combination of rafsiRNA-NeoPhectin and Taxotere® treatments resulted in 89% tumor growth inhibition compared to the control group; and 18-50% enhanced inhibition versus single agents (Figure 7I).

[0138] No morbidity or mortality was associated with rafsiRNA-NeoPhectin treatment. The marked knockdown of Raf-1 expression suggests that NeoPhectin-AT™ enhances the delivery and effectiveness of rafsiRNA *in vivo*. Administration of free rafsiRNA at BID schedule caused moderate inhibition of Raf-1 expression, and this may be attributed to rapid degradation or inactivation of free rafsiRNA or poor intracellular uptake of free rafsiRNA. Remarkable down regulation of Raf-1 protein expression was observed in tumor tissues in animals treated with rafsiRNA-NeoPhectin at QD or BID schedule as compared to animals treated with mismatch siRNA-NeoPhectin, suggesting that rafsiRNA specifically targets Raf-1. RafsiRNA-mediated silencing of Raf-1 was found to be associated with decreased expression of cyclin D1, a cyclin involved in regulation of cell cycle progression and neoplastic transformation. These data suggest that rafsiRNA could be explored as a next generation therapeutic agent administered alone or in combination with antineoplastic drugs for the treatment of human prostate or other cancer types.

### PREPARATORY EXAMPLES

#### Example 8A - Synthesis of cationic cardiolipin analog (11) [Figure 8A]

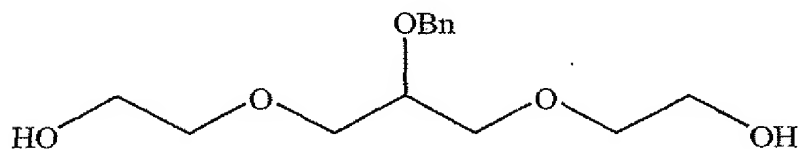
##### 1,3 Bis [(2-ethoxy tetrahydro-2H-pyran)]-2-benzyloxy-glycerol (7)



[00126] To a stirred suspension of sodium hydride (59.3 g, 1.48 mol, 60% in oil) in anhydrous DMF (300 mL) under argon atmosphere at 0°C, a solution of 2-benzyloxy 1,3-

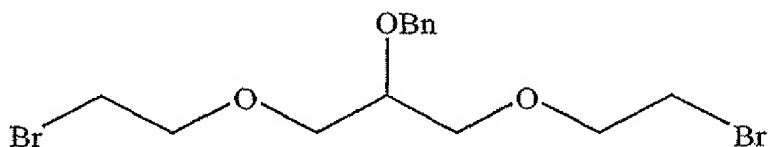
propanediol (5) (90 g, 0.49 mol) in DMF (700 mL) was added over a period of 2 h maintaining the temperature below 15°C. After stirring at room temperature for 2 h, 2-(2-bromoethoxy) tetrahydro-2*H*-pyran (6) (310 g, 1.48 mol) was added at 0°C over a period of 3 h maintaining the temperature below 10°C. The reaction mixture was stirred at room temperature for 12 h. The reaction mixture was cooled to 0°C and ice water was added very slowly to quench excess sodium hydride. The reaction mixture was concentrated under reduced pressure to remove maximum DMF and the crude solution was diluted with water (1 L) and extracted with ethyl acetate (2 x 500 mL). The organic layer was washed with aqueous saturated sodium chloride (500 mL) and dried over sodium sulfate. The solvent was concentrated under reduced pressure. The crude compound was purified by column chromatography over a silica gel (230-400 mesh) with 10-30% ethyl acetate in hexane to obtain 1,3-bis-[(2-ethoxy tetrahydro-2*H*-pyran)]-2-benzyloxy-glycerol (7) (154 g, 71%) as colorless oil. TLC (SiO<sub>2</sub>) hexane/ethyl acetate (3:2) *R<sub>f</sub>* ~ 0.40. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz): δ 1.41-1.82 (m, 12H), 3.41-3.98 (m, 17H), 4.61 (brs, 2H), 4.78 (s, 2 H, OCH<sub>2</sub>Ph), 7.24-7.45 (m, 5H, Ph-H).

3,7-Dioxa-5-benzyloxy-1,9-nonanediol (8)



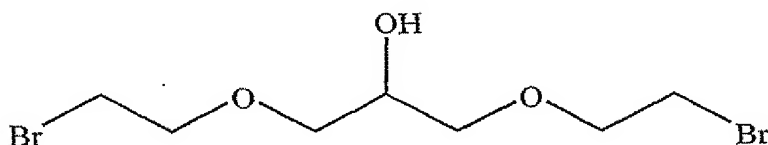
[00127] To a solution of 1,3-bis [(2-ethoxy tetrahydro-2*H*-pyran)]-2-benzyloxy-glycerol (7) (50 g, 0.11 mol) in methanol (500 mL) was added 1N HCl in ether (5 mL) and stirred at room temperature for 2 h. The reaction mixture was neutralized with sodium bicarbonate until the solution became neutral. The reaction mixture was filtered and concentrated under reduced pressure. The crude compound was dissolved in ethyl acetate (1 L), washed with water (100 mL) and dried over sodium sulfate. The organic layer was concentrated under reduced pressure and the crude compound was purified by column chromatography over a silica gel (70-230 mesh) eluting with ethyl acetate and followed by 5% methanol in ethyl acetate to obtain 3,7-dioxa-5-benzyloxy-1,9-nonanediol (8) (27 g, 88%) as colorless oil. TLC (SiO<sub>2</sub>) ethyl acetate *R<sub>f</sub>* ~ 0.10. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz): δ 3.01 (brs, 2H, OH), 3.50-3.81 (m, 13 H), 4.68 (s, 2 H, OCH<sub>2</sub>Ph), 7.21-7.42 (m, 5H, Ph-H).

1,9-Dibromo-3,7-dioxa-5-benzyloxy-nonane (9)



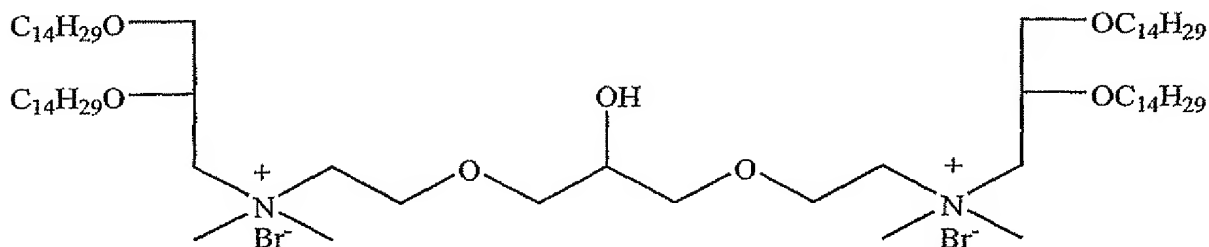
[00128] To a solution of 3,7-dioxa-5-benzyloxy-1,9-nonanediol (8) (27 g, 0.1 mol) in anhydrous dichloromethane (400 mL) under argon atmosphere at 0°C, was added triphenylphosphine (65.5 g, 0.25 mol) followed by carbon tetrabromide (79.4 g, 0.24 mol). The reaction mixture was stirred at 0°C for 2 h. The reaction mixture was diluted with water (300 mL) and the organic layer was separated, dried over sodium sulfate. The organic layer was concentrated under reduced pressure and crude compound was purified by column chromatography over a silica gel (70-230 mesh) with 20% ethyl acetate in hexane to obtain 1,9-dibromo-3,7-dioxa-5-benzyloxy-nonane (9) (36 g, 91%) as colorless oil. TLC (SiO<sub>2</sub>) hexane/ethyl acetate (3:2)  $R_f \sim 0.60$ . <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz):  $\delta$  3.45 (t,  $J = 5.5$  Hz, 4H, CH<sub>2</sub>Br), 3.60-3.67 (m, 4H, OCH<sub>2</sub>), 3.72-3.82 (m, 5H), 4.70 (s, 2H, OCH<sub>2</sub>Ph), 7.28-7.38 (m, 5H, Ph-H).

1,3-Bis-(2-bromoethoxy) propane-2-ol (10)



[00129] 1,9 Dibromo-3,7-dioxa-5-benzyloxy-nonane (9) (36 g, 90.90 mmol) was dissolved in ethanol (110 mL) and hydrogenated with 10% palladium on carbon (3.6 g) for 2 h at a pressure of 50 psi. After filtration of the catalyst, the solution was evaporated under reduced pressure. The crude material was subjected to silica-gel column chromatography (70-230 mesh) and eluted with 60% ethyl acetate in hexane to obtain 1,3-bis-(2-bromoethoxy) propane-2-ol (10) (26 g, 94%) as colorless oil. TLC (SiO<sub>2</sub>) hexane/ethyl acetate (1:1)  $R_f \sim 0.30$ . <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz):  $\delta$  2.56 (d,  $J = 4.5$  Hz, 1H, OH), 3.45 (t,  $J = 6.0$  Hz, 4H, CH<sub>2</sub>Br), 3.54-3.64 (m, 4H, OCH<sub>2</sub>), 3.82 (t,  $J = 5.7$  Hz, 4H, OCH<sub>2</sub>), 3.96-4.02 (m, 1H).

(R,S)-1,3-Bis-(1,2-ditetradecyloxypropyl-3-*N,N*-dimethyl-3-ethoxy ammonium bromide)propane-2-ol (11)

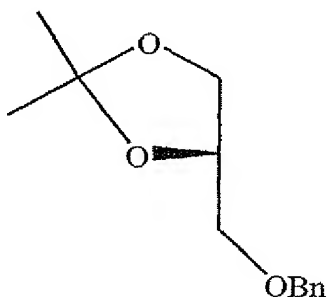


[00130] A solution of 1,2-bis-tetradecyloxy-3-dimethylamino propane (2) (50.2 g, 98.36 mmol) and 1,3-bis-(2-bromoethoxy) propane-2-ol (10) (10 g, 32.78 mmol) in anhydrous ethanol (600 mL) was refluxed at 78 - 80°C over a period of 5 days. The reaction mixture was

cooled and the solvent was evaporated to give a crude waxy solid. Hexane (400 mL) was added and stirred for 1 h. The solid was filtered and washed with hexane (6 x 100 mL). The compound was purified by recrystallization [ratio of compound/methanol/acetone (1:3:50)] and kept at  $-20^{\circ}\text{C}$  overnight. The separated solid was filtered and washed with cold acetone. The recrystallization was repeated two times to get analytically pure sample. The compound was dried for 24 h under vacuum and then over  $\text{P}_2\text{O}_5$  for 36 h to obtain cationic cardiolipin analog (**11**) (30 g, 69%) as a white solid. TLC ( $\text{SiO}_2$ ) methanol/chloroform (1:9)  $R_f \sim 0.11$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz):  $\delta$  0.88 (t,  $J = 6.7$  Hz, 12H), 1.25 (s, 88H), 1.52-1.71 (m, 8H), 3.41-3.68 (m, 29H), 3.95-4.19 (m, 14H), 4.66 (brs, 1H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 125 MHz):  $\delta$  13.92, 22.50, 25.89, 26.03, 29.12, 29.27, 29.32, 29.42, 29.48, 29.50, 29.54, 29.87, 31.74, 52.95, 53.02, 53.50, 65.02, 65.07, 66.57, 68.63, 68.84, 69.15, 71.80, 72.65, 73.29. IR ( $\text{cm}^{-1}$ ): 3323 (br), 2918 (s), 2873 (s), 1468 (s), 1123 (brs). ESI- MS 1248.6 [ $\text{M}+1\text{-Br}$ ], 584.3 [ $\text{M}+1\text{-2Br}/2$ ]. Mol. Formula  $\text{C}_{73}\text{H}_{152}\text{Br}_2\text{N}_2\text{O}_7$ ; elemental analysis; calcd. C: 65.93, H: 11.52, N: 2.11, Br: 12.02; found C: 65.93, H: 11.40, N: 2.11, Br: 11.99.

Example 8B - Synthesis of cationic cardiolipin analog (**19**) [Figure 8B]

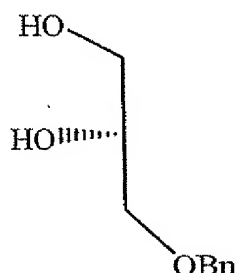
(*R*)-4-(Benzyloxymethyl)-2,2-dimethyl-1,3-dioxolane (**13**).



[00131] To a stirred suspension of sodium hydride (30.3 g, 0.75 mol, 60% in oil) in anhydrous tetrahydrofuran (500 mL) under argon atmosphere at  $0^{\circ}\text{C}$ , was added *R* (-)-2,2-dimethyl-1,3-dioxolane-4-methanol (**12**) (50 g, 0.37 mol) over period of 1 h maintaining the internal temperature below  $20^{\circ}\text{C}$ . After stirring at room temperature for 1 h, benzyl bromide (97.1 g, 0.56 mol) was added at  $0^{\circ}\text{C}$  over period of 1 h. After complete addition the reaction mixture was stirred for 14 h at room temperature. The reaction mixture was cooled to  $0^{\circ}\text{C}$ , added cold water very slowly, and this mixture was diluted with aqueous saturated ammonium chloride (500 mL). The aqueous layer was extracted with ethyl acetate (500 mL) and washed with water (300 mL). The organic layer was concentrated under reduced pressure and the crude product was obtained (121 g) (*R*)-4-benzyloxymethyl-2,2-dimethyl-1,3-dioxolane (**13**) as a syrup. TLC ( $\text{SiO}_2$ ) hexane/ethyl acetate (1:9)  $R_f \sim 0.26$  (The crude material was subjected to next step without purification.)  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz):  $\delta$  1.39

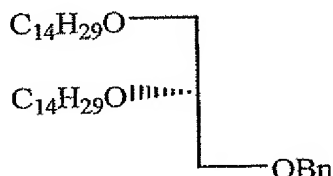
(s, 3H, CH<sub>3</sub>), 1.40 (s, 3H, CH<sub>3</sub>), 3.41-3.55 (m, 2H), 3.68-3.74 (m, 1H), 4.01-4.05 (m, 1H), 4.20-4.31 (m, 1H), 4.55 (brs, 2H, OCH<sub>2</sub>Ph), 7.25-7.35 (m, 5 H, Ph-H).

(S)-(-)-3-Benzyloxy-1,2-propanediol (14)



[00132] To a solution of (*R*)-4-benzyloxymethyl-2,2-dimethyl-1,3-dioxolane (13) (120 g) in methanol (700 mL) was added concentrated HCl (20 mL) and stirred at room temperature for 10 h. The reaction mixture was neutralized with sodium bicarbonate until the solution became neutral. The reaction mixture was filtered and concentrated under reduced pressure. The crude compound was dissolved in dichloromethane (600 mL) and organic layer was separated and dried over sodium sulfate. The organic layer was concentrated under reduced pressure. The crude compound was purified by column chromatography over a silica gel (230-400 mesh) with 10-20% ethyl acetate in hexane as eluent followed by 10% methanol in ethyl acetate to obtain (*S*)-(-)-3-benzyloxy-1,2-propanediol (14) (64 g, 93%) as syrup. TLC (SiO<sub>2</sub>) ethyl acetate *R<sub>f</sub>* ~ 0.44. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz): δ 3.42-3.61 (m, 4H), 3.79-3.83 (m, 3H), 4.47 (s, 2H, OCH<sub>2</sub>Ph), 7.23-7.32 (m, 5H, Ph-H).

(S)-1,2-Bis-tetradecyloxy-3-*O*-benzylpropane (15).

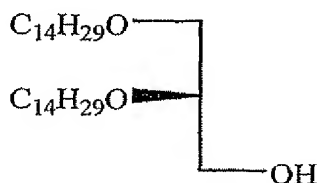


[00133] To a stirred suspension of sodium hydride (54.5 g, 1.36 mol, 60% in oil) in anhydrous DMF (220 mL) under argon atmosphere at 0°C, was added a solution of (*S*)-(-)-3-benzyloxy-1,2-propanediol (14) (62 g, 0.34 mol) in DMF (400 mL) over period of 1 h maintaining the internal temperature below 20°C. After stirring at room temperature for 2 h, tetradecyl bromide (377.4 g, 1.36 mol) was added at 0°C over period of 2 h. After complete addition, the reaction mixture was stirred for 2 h at room temperature and the temperature was gradually increased to 70°C, then stirred for 5 h. The reaction mixture was cooled to 0°C, added cold water very slowly and diluted with aqueous saturated ammonium chloride (500 mL). The aqueous layer was extracted with ethyl acetate (1 L) and washed with water (3 x 1



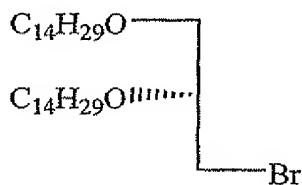
L). The organic layer was concentrated under reduced pressure and the crude product was purified by column chromatography over a silica gel (70-230 mesh) eluting with 2-10% ethyl acetate in hexane to obtain (*S*)-1,2-bis-tetradecyloxy-3-*O*-benzylpropane (**15**) (146 g, 75%) as colorless oil. TLC (SiO<sub>2</sub>) hexane/ethyl acetate (1:9) *R*<sub>f</sub> ~ 0.53. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz): δ 0.88 (t, *J* = 6.7 Hz, 6H), 1.25 (brs, 44H), 1.49-1.58 (m, 4H), 3.39-3.60 (m, 9H), 4.54 (m, 2H, OCH<sub>2</sub>Ph), 7.23-7.32 (m, 5H, Ph-H).

(*R*)-1,2-Bis-tetradecyloxy-propan-3-ol (**16**)



[00134] A solution of (*S*)-1,2-bis-tetradecyloxy-3-*O*-benzylpropane (**15**) (70 g, 0.12 mmol) was dissolved in ethyl acetate (280 mL) and hydrogenated with 10% palladium (3 g) for 12 h at a pressure of 50 psi. After filtration of the catalyst, the solution was evaporated under reduced pressure. The residue was dissolved in hot ethanol (500 mL) and kept at -20°C overnight. The separated solid was filtered and dried under vacuum to obtain (*R*)-1,2-bis-tetradecyloxy-propan-3-ol (**16**) (54 g, 92%) as a white solid. TLC (SiO<sub>2</sub>) hexane/ethyl acetate (1:9) *R*<sub>f</sub> ~ 0.17. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz): δ 0.88 (t, *J* = 6.7 Hz, 6H), 1.25 (brs, 48H), 1.59-1.57 (m, 4H), 2.2 (t, *J* = 5.7 Hz, 1H, OH), 3.37-3.73 (m, 9H).

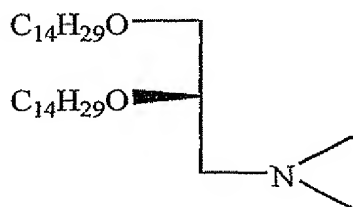
(*S*)-1,2-Bis-tetradecyloxy-3-bromopropane (**17**)



[00135] To a solution of (*R*)-1,2-bis-tetradecyloxy-propan-3-ol (**16**) (52 g, 0.1 mol) in anhydrous dichloromethane (280 mL) under argon atmosphere at 0°C was added triphenylphosphine (35.1 g, 0.13 mol). A solution of carbon tetrabromide (46.2 g, 0.13 mol) in dichloromethane (240 mL) was added to the reaction mixture dropwise a period of 1 h. and further stirred at 0°C for 3 h. The reaction mixture was diluted with water (500 mL) and the organic layer was separated, dried over sodium sulfate. The organic layer was concentrated under reduced pressure and the crude compound was purified by column chromatography over a silica gel (230-400 mesh) with 1-5 % ethyl acetate in hexane to obtain (*S*)-1,2-bis-tetradecyloxy-3-bromopropane (**17**) (53 g, 90%) as colorless oil. TLC (SiO<sub>2</sub>) hexane/ethyl

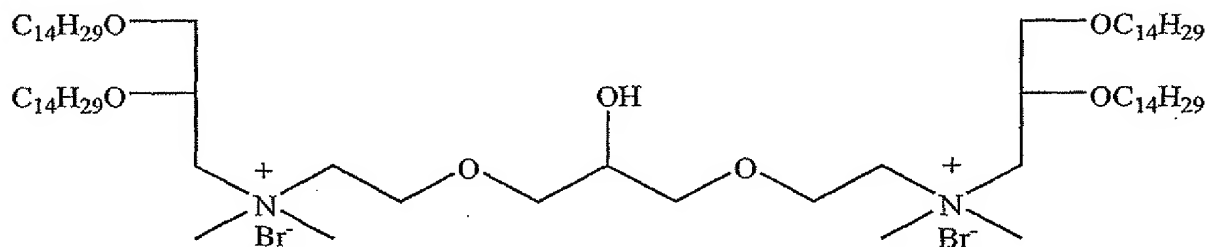
acetate (1:9)  $R_f \sim 0.72$ .  $^1\text{H NMR}$  ( $\text{CDCl}_3$ , 300 MHz):  $\delta$  0.88 (t,  $J = 6.7$  Hz, 6H), 1.25 (brs, 48H), 1.51-1.61 (m, 4H), 3.39-3.61 (m, 9H).

(R)-1,2-Bis-tetradecyloxy-3-dimethylamino propane (18)



[00136] (S)-1,2-Bis-tetradecyloxy-3-bromopropane (17) (50 g, 0.09 mol) was dissolved in a 2M methanolic solution of dimethylamine (400 mL) in a screw-top pressure bottle. The pressure bottle was sealed and heated in an oil bath with stirring at 88-90°C for 60 h. The pressure bottle was cooled and the solution was concentrated under reduced pressure. The crude residue was dissolved in ethyl acetate (500 mL) and washed with water (500 mL). The organic layer was concentrated under reduced pressure and purified by column chromatography over a silica gel (230- 400 mesh) with 5- 20% ethyl acetate in hexane as eluent to obtain (R)-1,2-bis-tetradecyloxy-3-dimethylamino propane (18) (41 g, 88%) as light colored oil. TLC ( $\text{SiO}_2$ ) methanol/chloroform (1:9)  $R_f \sim 0.51$ .  $^1\text{H NMR}$  ( $\text{CDCl}_3$ , 300 MHz):  $\delta$  0.88 (t,  $J = 6.8$  Hz, 6H), 1.25 (s, 44 H), 1.51 - 1.58 (m, 4 H), 2.25 (s, 6H, N- $\text{CH}_3$ ), 2.37 (t,  $J = 4.6$  Hz, 2H, N- $\text{CH}_2$ ), 3.41-3.62 (m, 7H).

(R)-1,3-Bis-(1,2-ditetradecyloxypropyl-3-*N,N*-dimethyl-3-ethoxy ammonium bromide)propane-2-ol (19)

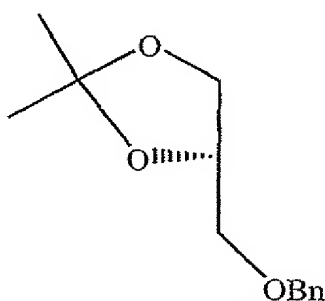


[00137] A solution of (R)-1,2-bis-tetradecyloxy-3-dimethylamino propane (18) (35.6 g, 69.8 mmol) and 1,3-bis-(2-bromoethoxy) propane-2-ol (10) (7.1 g, 23.2 mmol) in anhydrous ethanol (430 mL) was refluxed at 78 - 80°C over a period of 5 days. The hot reaction mixture was transferred to erlenmeyer flask and added acetone (4.3 L) dropwise over period of 2 h. The mixture was keep at -20°C over night. The solid was filtered and washed with cold acetone (500 mL) to obtain a colorless white solid (28 g). The crude solid was purified by recrystallization in the mixture of warm methanol (140 mL): acetone (1.4 L) and then stored at -20°C overnight. The solid was separated, filtered and washed with cold acetone (300 mL). The recrystallization was repeated two times to get analytically pure sample. The

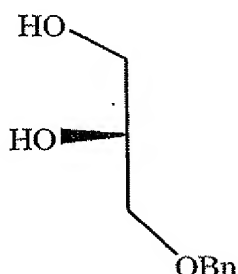
compound was dried for 24 h and then over  $P_2O_5$  for 36 h under vacuum to obtain (*R*)-cationic cardiolipin analog (**19**) (24 g, 78%) as a white solid. TLC ( $SiO_2$ ) methanol/chloroform (1:9)  $R_f \sim 0.13$ .  $^1H$  NMR ( $CDCl_3$ , 500 MHz):  $\delta$  0.88 (t,  $J = 6.7$  Hz, 12H), 1.25 (s, 88H), 1.52-1.71 (m, 8H), 3.41-3.68 (m, 30H), 3.95-4.19 (m, 13H), 4.63 (brs, 1H, OH).  $^{13}C$  NMR ( $CDCl_3$ , 125 MHz):  $\delta$  13.94, 22.52, 25.90, 26.04, 29.20, 29.28, 29.33, 29.44, 29.50, 29.52, 29.55, 29.88, 31.76, 52.99, 53.07, 53.49, 65.01, 65.07, 66.56, 68.66, 68.84, 69.17, 71.82, 72.62, 72.64, 73.30, 73.28. IR ( $cm^{-1}$ ): 3409 (br, OH), 2918 (s), 2873 (s), 1468 (s), 1124 (br s). ESI-MS 1248.5 [ $M+1-Br$ ], 584.2 [ $M+1-2Br/2$ ] Mol. Formula  $C_{73}H_{152}Br_2N_2O_7$ ; elemental analysis; calcd. C: 65.93, H: 11.52, N: 2.11, Br: 12.02; found C: 65.65, H: 11.49, N: 2.13, Br: 12.17.

Example 8C - Synthesis of cationic cardiolipin analog (27) [Figure 8C]

(*S*)-4-(Benzyloxymethyl)-2,2-dimethyl-1,3-dioxolane (21).



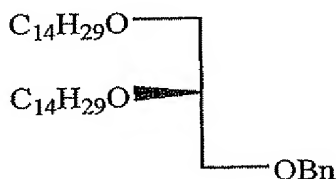
[00138] To a stirred suspension of sodium hydride (22.7 g, 0.57 mol, 60% in oil) in anhydrous tetrahydrofuran (350 mL) under argon atmosphere at  $0^\circ C$ , was added *S* (-)-2,2-dimethyl-1,3-dioxolane-4-methanol (**20**) (50 g, 0.37 mol) over period of 1 h maintaining the internal temperature below  $20^\circ C$ . After stirring at room temperature for 1 h, benzyl bromide (97.1 g, 0.56 mol) was added at  $0^\circ C$  over period of 1 h. After complete addition the reaction mixture was stirred for 18 h at room temperature. The reaction mixture was cooled to  $0^\circ C$ , added few drops of ice water (20 mL) very slowly, and this mixture was diluted with aqueous saturated ammonium chloride (500 mL). The aqueous layer was extracted with ethyl acetate (750 mL). The organic layer was concentrated under reduced pressure and the crude product was obtained (83 g) of (*S*)-4-benzyloxymethyl-2,2-dimethyl-1,3-dioxolane (**21**) as a syrup. TLC ( $SiO_2$ ) hexane/ethyl acetate (1:9)  $R_f \sim 0.26$ .

(R)-(-)-3-Benzoyloxy-1,2-propanediol (22)

[00139] To a solution of (*S*)-4-(benzyloxymethyl)-2,2-dimethyl-1,3-dioxolane (21) (83 g) in methanol (800 mL) was added concentrated HCl (20 mL) and stirred at room temperature for 15 h. The reaction mixture was neutralized with sodium bicarbonate until the solution became neutral. The reaction mixture was filtered and concentrated under reduced pressure. The crude compound was dissolved in dichloromethane (200 mL) and organic layer was separated, dried over sodium sulfate and concentrated under reduced pressure. The crude compound was purified by column chromatography over a silica gel (230-400 mesh) with 10-20% ethyl acetate in hexane as eluent followed by 10% methanol in ethyl acetate to obtain (*R*)-(-)-3-benzyloxy-1,2-propanediol (22) (65 g, 94%) as syrup. TLC (SiO<sub>2</sub>) ethyl acetate *R<sub>f</sub>* ~ 0.44. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz): δ 3.42-3.61 (m, 4H), 3.75-3.99 (m, 3H), 4.47 (s, 2H, OCH<sub>2</sub>Ph), 7.22-7.32 (m, 5H, Ph-H).

(R)-1,2-Bis-tetradecyloxy-3-*O*-benzylpropane (23)

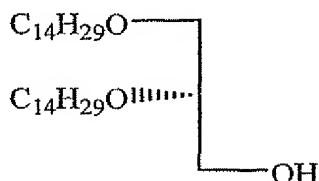
[00140]



[00141] To a stirred suspension of sodium hydride (38.4 g, 0.96 mol, 60% in oil) in anhydrous DMF (200 mL) under argon atmosphere at 0°C, was added a solution of (*R*)-(-)-3-benzyloxy-1,2-propanediol (22) (35 g, 0.19 mol) in DMF (150 mL) over period of 1 h maintaining the internal temperature below 20°C. After stirring at room temperature for 2 h, tetradecyl bromide (266 g, 0.97 mol) was added at 0°C over period of 2 h. After complete addition, the reaction mixture was stirred at room temperature for 2 h and the temperature was gradually increased to 60°C, then stirred for 20 h. The reaction mixture was cooled to 0°C, added few drops of ice water very slowly and diluted with aqueous saturated ammonium chloride (500 mL). The aqueous layer was extracted with hexane (3x500 mL) and washed with water (500 mL) and brine (500 mL). The organic layer was concentrated under reduced pressure and the crude product was purified by column chromatography over a silica gel (70-

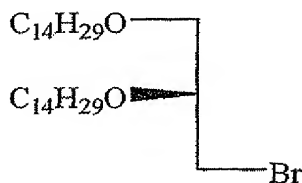
230 mesh) eluting with 2-10 % ethyl acetate in hexane to obtain (*R*)-1,2-bis-tetradecyloxy-3-*O*-benzylpropane (**23**) (88 g, 80%) as a colorless oil. TLC (SiO<sub>2</sub>) hexane/ethyl acetate (1:9)  $R_f \sim 0.53$ . <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz):  $\delta$  0.88 (t,  $J = 6.6$  Hz, 6H), 1.25 (brs, 44H), 1.49-1.58 (m, 4H), 3.39-3.60 (m, 9H), 4.54 (m, 2H, OCH<sub>2</sub>Ph), 7.23-7.32 (m, 5H, Ph-H).

(*S*)-1,2-Bis-tetradecyloxy-propan-3-ol (**24**)



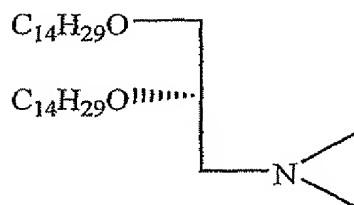
[00142] (*R*)-1,2-bis-tetradecyloxy-3-*O*-benzylpropane (**23**) (83.8 g, 0.14 mol) was dissolved in ethyl acetate (450 mL) and hydrogenated with 10% palladium on carbon (3.1 g) for 12 h at a pressure of 50 psi. After filtration of the catalyst, the solution was evaporated under reduced pressure. The residue was dissolved in hot hexane (200 mL) and kept at  $-20^\circ\text{C}$  overnight. The separated solid was filtered and dried to afford (*S*)-1,2-bis-tetradecyloxy-propan-3-ol (**24**) (64.8 g, 92%) as a white solid. TLC (SiO<sub>2</sub>) hexane/ethyl acetate (1:9)  $R_f \sim 0.17$ . <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz):  $\delta$  0.88 (t,  $J = 6.7$  Hz, 6H), 1.25 (brs, 44H), 1.51-1.57 (m, 4H), 2.22 (t,  $J = 5.2$  Hz, 1H, OH), 3.40-3.73 (m, 9H).

(*R*)-1,2-Bis-tetradecyloxy-3-bromopropane (**25**)



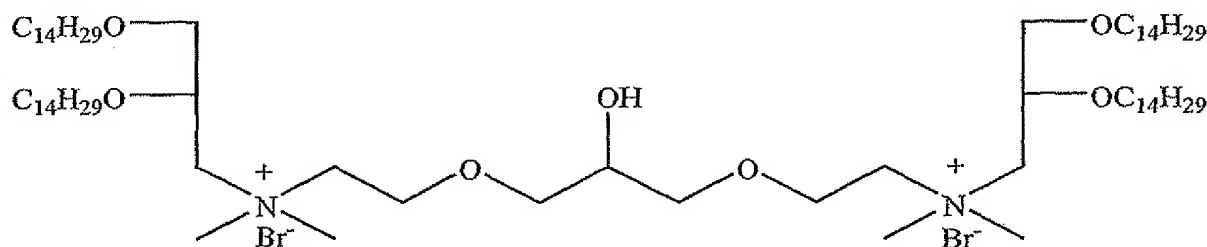
[00143] To a solution of (*S*)-1,2-bis-tetradecyloxy-propan-3-ol (**24**) (36.8 g, 59.0 mmol) in anhydrous dichloromethane (375 mL) under argon atmosphere at  $0^\circ\text{C}$  was added triphenylphosphine (27.9 g, 106.0 mmol). A solution of carbon tetrabromide (36.2 g, 109.0 mmol) in dichloromethane (60 mL) added dropwise to reaction mixture over period of 1 h. The reaction mixture was further stirred at  $0^\circ\text{C}$  for 2 h. and then diluted with water (3x300 mL). The organic layer was separated, dried over sodium sulfate, and concentrated under reduced pressure. The crude compound was purified by column chromatography over a silica gel (230-400 mesh) with 1-5 % ethyl acetate in hexane to obtain (*R*)-1,2-bis-tetradecyloxy-3-bromopropane (**25**) (37.8 g, 87%) as colorless oil. TLC (SiO<sub>2</sub>) hexane/ethyl acetate (1:9)  $R_f \sim 0.72$ . <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz):  $\delta$  0.88 (t,  $J = 6.7$  Hz, 6H), 1.25 (brs, 44H), 1.50-1.60 (m, 4H), 3.40-3.68 (m, 9H).

(*S*)-1,2-Bis-tetradecyloxy-3-dimethylamino propane (**26**)



[00144] (*R*)-1,2-Bis-tetradecyloxy-3-bromopropane (**25**) (36.7 g, 0.07 mol) was dissolved in a 2M methanolic solution of dimethylamine (450 mL) in a screw-top pressure bottle. The pressure bottle was sealed and heated in an oil bath with stirring at 92°C for 75 h. The pressure bottle was cooled and the solution was concentrated under reduced pressure. The crude residue was dissolved in ethyl acetate (500 mL) and washed with water (500 mL). The organic layer was concentrated under reduced pressure and purified by column chromatography over a silica gel (230- 400 mesh) with 5- 20% ethyl acetate in hexane as eluent to obtain (*S*)-1,2-bis-tetradecyloxy-3-dimethylamino propane (**26**) (28.7 g, 80%) as light colored oil. TLC (SiO<sub>2</sub>) methanol/chloroform (2:8) *R<sub>f</sub>* ~ 0.66. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz): δ 0.88 (t, *J* = 6.7 Hz, 6H), 1.25 (s, 44 H), 1.51 - 1.57 (m, 4 H), 2.15, 2.25 (s, 6H, N-CH<sub>3</sub>), 2.34-2.36 (m, 2H, N-CH<sub>2</sub>), 3.38-3.61(m, 7H).

(*S*)-1,3-Bis-(1,2-ditetradecyloxypropyl-3-*N,N*-dimethyl-3-ethoxy ammonium bromide)propane-2-ol (**27**)



[00145] A solution of (*S*)-1,2-bis-tetradecyloxy-3-dimethylamino propane (**26**) (22.5 g, 43.9 mmol) and 1,3-bis-(2-bromoethoxy) propane-2-ol (**10**) (4.48 g, 14.6 mmol) in anhydrous ethanol (220 mL) was refluxed at 78 - 80°C over a period of 5 days. The hot solution was transferred into a erlenmeyer flask and acetone (2.5 L) was added dropwise while stirring the solution. The flask was stored in the freezer (-25°C) for 15 hrs. The white solid was filtered and washed with cold acetone (100 mL). The product was dissolved in chloroform (100 mL) and acetone (1 L) was added. The flask was stored at -25°C for 15 hours. The separated white solid was filtered and washed with cold acetone (100 mL). The recrystallization procedure was repeated three times. The product was triturated with hexane (1 L) and filtered and dried over P<sub>2</sub>O<sub>5</sub> under high vacuum for 24h. to obtain (*S*)-cationic cardiolipin

analog (27) (15.5 g, 80%) as a white solid. TLC (SiO<sub>2</sub>) methanol/chloroform (1:9) R<sub>f</sub> ~ 0.13. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz): δ 0.88 (t, *J* = 6.6 Hz, 12H), 1.25 (s, 88H), 1.52-1.71 (m, 8H), 3.40-3.71 (m, 36H), 3.92-4.11 (m, 7H), 4.74 (d, *J* = 5.7 Hz, 1H, OH). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 125 MHz): δ 13.91, 22.48, 25.86, 26.0, 29.16, 29.24, 29.30, 29.40, 29.46, 29.48, 29.51, 29.84, 31.72, 52.93, 53.01, 53.46, 64.98, 66.51, 86.63, 68.8, 69.12, 71.77, 72.61, 73.26. IR (cm<sup>-1</sup>): 3397 (br, OH), 2917 (s), 1467 (s), 1122 (br s). ESI-MS 1248.5 [M+1-Br], 584.4 [M+1-2Br/2].

**[0139]** All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

**[0140]** The use of the terms “a” and “an” and “the” and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,” “having,” “including,” and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to,”) unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

**[0141]** Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

**Table 1A**

Formulation Lot #	Molar Ratio		Lipid Concentration		Liposome Size (nm)		pH
	PCL-2/CHOL	PCL-2/DOPE	PCL-2 (mM)	Total Lipids (mg/mL)	Mean	D99	
PDM-GT-03-001	1 : 1		10	17.14	144.2	259.4	7.54
PDM-GT-03-002	3 : 2		10	15.85	173.8	290.2	7.45
PDM-GT-03-003	2 : 3		10	19.07	128.5	230.0	7.44
PDM-GT-03-004	2 : 1		10	15.20	163.3	311.4	7.64
PDM-GT-03-005		1 : 1	10	20.71	105.7	211.7	7.37
PDM-GT-03-006		2 : 1	10	16.99	101.6	182.0	7.28
PDM-GT-03-007		1 : 2	10	28.15	114.8	208.7	7.32
PDM-GT-03-008		3 : 2	10	18.23	109.2	196.0	7.46
PDM-GT-03-009		2 : 3	10	24.43	117.5	219.9	7.29

**Table 1B**

Formulation Lot#	Molar Ratio	Lipid Concentration		Liposome Size (nm)		pH
	PCL-2/DOPE	PCL-2 (mg/mL)	Total Lipids (mg/mL)	Mean	D99	
001N0104	1:2	0.47	1 mg	115.6	224.6	4.3

**Table 2: One Month Stability data for GMP batch**

Test	Specification	Initial	Storage Conditions	
			5°C (inverted)	25°C/60%RH (inverted)
Appearance	White Suspension	Conforms	Conforms	Conforms
pH	Report	5.0	5.1	5.0
rafAON	90-110%	107%	102.5%	103.0%
DOPC	70-110%	92.2%	93.8%	93.1%
Cholesterol	70-110%	93.5%	94.9%	94.7%
PCL-2	70-110%	87.1%	89.3%	90.8%
Mean Particle Size	< 400 nm	138.6nm	135.7nm	135.1nm
rafAON Entrapment	> 85%	100%	100.0%	100.0%
Efficiency				



**Table 3A. Stability of lamellar complex formulation of c-rafAON**

TIME (Month)	Storage conditions	Particle size (nm)		Entrapment Efficiency (%)
		Mean	99 percentile <	
Initial	—	162	294	98.8
2 month	2-8°C	162	287	98.7

**Table 3B. Stability of lamellar complex formulation of c-rafAON**

TIME (month)	Storage conditions	c-raf AON (% of initial concentration)	DOPC (% of initial concentration)	PCL-2 (% of initial concentration)
Initial	—	100	100	100
2 month	2-8°C	96	98	97

**Table 3C: Results of Liposomal rafAON (LrafAON) 8-fold diluted with 5% dextrose solution**

[illegible]

**Table 6: Toxicity studies of the CCLA-based liposome****Single-dose toxicity (I.V. x 1)**

Transfection reagents	Dose (mg/kg)	Mortality rate (%)
CCLA-based liposome	100	0
<i>In Vivo</i> GeneSHUTTLE™	100	66.7

**Multiple-dose toxicity (I.V. x 3)**

Transfection reagents	Cumulative Dose (mg/kg/day)	Mortality rate (%)
CCLA-based liposome	50	0
	75	0
	100	33.3

What is claimed is:

1. A composition comprising 1,3-Bis-(1,2-bis-tetradecyloxy-propyl-3-dimethylethoxyammoniumbromide)-propane-2-ol (PCL-2) and further comprising at least one lipid selected from the group of lipids consisting of cholesterol, dioleoylphosphatidylethanolamine (DOPE), 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), alpha tocopheryl acid succinate and any other phosphatidylcholine.]
2. The composition of claim 1, consisting of DOPC, PCL-2, and cholesterol.
3. The composition of claim 2, having a percent molar ratio of DOPC:cholesterol:PCL-2 of between about (50-65):(25-35):(5-20).
4. The composition of claim 2 or 3, further comprising D-alpha tocopheryl acid succinate
5. The composition of claim 4, wherein the D-alpha tocopheryl acid succinate is present at between about 0.1 wt % and about 1 wt %.
6. The composition of claim 1, consisting of PCL-2 and cholesterol.
7. The composition of claim 6, wherein the molar ratio of PCL-2 and cholesterol is between 1:3 and 6:1.
8. The composition of claim 1, consisting of PCL-2 and DOPE.
9. The composition of claim 8, wherein the molar ratio of PCL-2 and DOPE is between 1:3 and 3:1.
10. The composition of claim 9, wherein the molar ratio of PCL-2 and DOPE is 1:2.
11. The composition of any of claims 6-10, wherein the total lipid concentration is between about 1.0 mg/mL and about 60 mg/mL.
12. The composition of any of claims 1-11, wherein the concentration of PCL-2 is about 10 mM.
13. The composition of any of claims 1-12, further comprising a sugar.
14. The composition of claim 13, wherein the sugar is sucrose.

15. The composition of claim 13 or 14, wherein the sugar is present at about 5 wt% to about 30 wt%.
16. The composition of any of claims 1-15, further comprising a carrier.
17. The composition of claim 16, wherein the carrier is water.
18. The composition of claim 16, wherein the carrier is a physiologically-compatible buffer.
19. The composition of any of claims 1-18, wherein a portion of the PCL-2 is part of an emulsion.
20. The composition of any of claims 1-18, wherein a portion of the PCL-2 is present within liposomes.
21. The composition of claim 20, wherein the mean size of the liposomes is between about 50 nm and about 200 nm.
22. The composition of claim 20 or 21, wherein 99% of the liposomes have a diameter less than between about 170 nm and about 500 nm.
23. The composition of any of claims 1-22, having a pH of between about 3 and about 8.
24. The composition of any of claims 1-22, having a pH of between about 7 and about 8.
25. The composition of any of claims 1-24, further comprising at least one active agent.
26. The composition of claim 25, wherein the active agent is a polynucleotide.
27. The composition of claim 26, wherein the polynucleotide encodes a therapeutic polypeptide.
28. The composition of claim 27, wherein the therapeutic polypeptide comprises immunogenic peptides.
29. The composition of claim 26, wherein the polynucleotide is DNA.

30. The composition of claim 29, wherein the DNA is an oligodeoxyribonucleotide.

31. The composition of claim 30, wherein the respective charge ratio of cationic cardiolipin PCL-2:oligodeoxyribonucleotide ranges from (1.0-4.0): 1.

32. The composition of claim 30, wherein said respective total lipids to oligodeoxyribonucleotide molar amounts ranges from (10-200):1.

33. The composition of claim 30, wherein the oligodeoxyribonucleotide contains at least one phosphothioate modification.

34. The composition of claim 30, wherein the oligodeoxyribonucleotide ranges in size from 10 to 40 nucleotides.

35. The composition of claim 34, wherein the oligodeoxyribonucleotide ranges in size from 15 to 25 nucleotides.

36. The composition according to any of claims 30-35, which comprises liposomes and wherein between 60-80% of the oligodeoxyribonucleotide is complexed with cationic lipids in the inner core of the liposomes.

37. The composition of any of claims 30-36, wherein the oligodeoxyribonucleotide has a sequence comprising 5'-GTGCTCCATTGATGC-3' (SEQ ID NO: 1).

38. The composition of claim 26, wherein the polynucleotide is RNA.

39. The composition of claim 38, wherein the RNA is siRNA.

40. The composition of any of claims 26-39, wherein the polynucleotide hybridizes to a human mRNA.

41. The composition of any of claims 26-39, wherein the polynucleotide hybridizes to a plant mRNA.

42. The composition of any of claims 26-39, wherein the polynucleotide hybridizes to a plant parasitic or fungal mRNA.

43. The composition of any of claims 40-42, wherein the polynucleotide inhibits the expression of the gene upon binding to the mRNA.

44. The composition of claim 40, wherein the mRNA is an oncogene.
45. The composition of claim 44, wherein the oncogene is selected from the group of oncogenes consisting of ras, raf cot, mos, myc.
46. The composition of claim 44 or 45, wherein the oncogene is c-raf-1.
47. Use of the composition of any of claims 1-46 for cosmetics.
48. Use of the composition of any of claims 1-46 as a vaccine.
49. Use of the composition of any of claims 1-46 for agricultural purposes.
50. A method of inhibiting the growth of neoplastic cells comprising administering the composition of any of claims 40-46 to neoplastic cells under conditions such that said polynucleotide enters said cells, whereby the polynucleotide inhibits the expression of said oncogene to which it is targeted within said neoplastic cells.
51. The method of claim 50, wherein said cells are *in vitro*.
52. The method of claim 50, wherein said cells are *in vivo*.
53. A method of treating a patient suffering from a neoplastic disease comprising administering the composition of any of claims 40, 43, 44, 45 or 46 to said patient under conditions such that said polynucleotide enters neoplastic cells within said patient, whereby the polynucleotide inhibits the expression of said oncogene to which it is targeted within said neoplastic cells.
54. The method of claim 53, wherein the patient is a vertebrate animal.
55. The method of claim 54, wherein the vertebrate animal is a human.
56. The method of any of claims 53-55, wherein said neoplastic disease comprises cancer.
57. The method of any of claims 53-56, wherein said neoplastic cells are within a tumor.
58. The method of any of claims 53-56, wherein said neoplastic cells are metastasized cells.

59. The method of any of claims 53-58, wherein the composition is administered adjunctively with a second antineoplastic agent.
60. The method of claim 59, wherein the composition is administered prior to, concurrently with, or after the second antineoplastic agent.
61. The method of claim 59 or 60, wherein the antineoplastic agent is radiation.
62. The method of claim 59 or 60, wherein the antineoplastic agent is a chemotherapeutic agent.
63. The method of claim 59 or 60, wherein the antineoplastic agent is docetaxel.
64. A method for validating a target gene, comprising (a) administering to a cell a composition comprising a cationic liposome and an RNAi, whereby the RNAi enters the cell inhibits the expression of a gene within the cell and (b) assaying for the inhibition of the gene.
65. The method of claim 64, wherein the composition is the composition of any of claims 1-46.
66. A fluorescent cationic cardiolipin analogue.
67. The fluorescent cationic cardiolipin analogue of claim 66, comprising a cationic cardiolipin molecule conjugated to a fluorescent/ luminescent moiety.
68. The fluorescent cationic cardiolipin analogue of claim 67, wherein the cationic cardiolipin molecule is PCL-2.
69. A luminescent cationic cardiolipin analogue.
70. The luminescent cationic cardiolipin analogue of claim 69, comprising a cationic cardiolipin molecule conjugated to a fluorescent/ luminescent moiety.
71. The luminescent cationic cardiolipin analogue of any of claims 69-70, wherein the cationic cardiolipin molecule is PCL-2.
72. A composition comprising the fluorescent/ luminescent cationic cardiolipin analogue of any of claims 66-71 and a physiologically-acceptable carrier.
73. A liposomal composition comprising the fluorescent/ luminescent cationic cardiolipin analogue of any of claims 66-71.

74. The liposomal composition of claim 73, further comprising an additional lipid.
75. The liposomal composition of claim 74, further comprising a physiologically acceptable carrier.
76. A method of tracking the migration of a lipid substance within an animal comprising (a) introducing the composition of any of claims 72-75 into an animal, (b) causing the fluorescent/ luminescent cationic cardiolipin analogue to fluoresce or luminate, and (c) observing the position of fluorescence or luminescence.
77. The method of claim 76, wherein steps (b) and (c) are repeated one or more times.
78. The use of a composition of any of claims 1-46 as a transfection agent.
79. A method of transfecting a cell with a polynucleotide, comprising exposing the cell to the composition of any of claims 1-46 under conditions sufficient for the polynucleotide present within the composition to enter the cell.
80. The method of claim 79, wherein the cell is *in vitro*.
81. The method of claim 79, wherein the cell is *in vivo*.
82. The composition of claim 39, wherein the siRNA comprises the following sequence:
- SEQ ID NO: 1  
5'-UGGAAUGAGCUUACAUGACdTdT-3'  
3'-dTdTACCUUACUCGAAUGUACUG-5'
83. The composition of claim 39, wherein the siRNA comprises the following sequence:
- SEQ ID NO: 2  
5'-GCACGCUUAGAUUGGAACAdTdT-3'  
3'-dTdTTCGUGCGAAUCUAACCUUGU-5'



84. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 3

5'-UGCGUCGGAUGCGAGAAUCdTdT-3'  
3'-dTdTACGCAGCCUACGCUCUUAG-5'

85. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 4

5'-UCCGGAUGCAGGAUGACAAdTdT-3'  
3'-dTdTAGGCCUACGUCCUACUGUU-5'

86. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 5

5'-UCAACAGGAGCGCCUCUGAdTdT-3'  
3'-dTdTAGUUGUCCUCGCGGAGACU-5'

87. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 6

5'-UGCUUGCACGCUGACUACAdTdT-3'  
3'-dTdTACGAACGUGCGACUGAUGU-5'

88. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 7

5'-AUUCCUGCUCAAUGGAUUUdTdT-3'  
3'-dTdTUAAGGACGAGUUACCUAAA-5'

89. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 8

5'-GCUGCAUCAAUGGAGCACAdTdT-3'

3'-dTdTTCGACGUAGUUACCUCGUGU-5'

90. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 9

5'-CUGCAUCAAUGGAGCACAUdTdT-3'

3'-dTdTTCGACGUAGUUACCUCGUGUA-5'

91. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 10

5'-UGCAUCAAUGGAGCACAUAdTdT-3'

3'-dTdTACGUAGUUACCUCGUGUAU-5'

92. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 11

5'-GCAUCAAUGGAGCACAUACdTdT-3'

3'-dTdTTCGUAGUUACCUCGUGUAUG-5'

93. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 12

5'-GCUGCAUCAAUGGAGCACAUU-3'

3'-UUCGACGUAGUUACCUCGUGU-5'

94. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 13

5'-CUGCAUCAAUGGAGCACAUUU-3'

3'-UUGACGUAGUUACCUCGUGUA-5'

95. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 14

5'-UGCAUCAAUGGAGCACAUUU-3'

3'-UUACGUAGUUACCUCGUGUAU-5'

96. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 15

5'-GCAUCAAUGGAGCACAUACUU-3'

3'-UUCGUAGUUACCUCGUGUAUG-5'

97. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 16

5'-GCUGCAUCAAUGGAGCACAUAdTdT-3'

3'-dTdTTCGACGUAGUUACCUCGUGUAU-5'

98. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 17

5'-AAGCUGCAUCAAUGGAGCACAdTdT-3'

3'-dTdTUUCGACGUAGUUACCUCGUGU-5'

99. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 18

5'-GCAUCAAUGGAGCACAUACAGdTdT-3'

3'-dTdTTCGUAGUUACCUCGUGUAUGUC-5'

100. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 19

5'-CUGCAUCAAUGGAGCACAUACdTdT-3'

3'-dTdTGACGUAGUUACCUCGUGUAUG-5'

101. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 20

5'-AAGCUGCAUCAAUGGAGCACAUACdTdT-3'

3'-dTdTUUCGACGUAGUUACCUCGUGUAUG-5'

102. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 21

5'-GCUGCAUCAAUGGAGCACAUACAGdTdT-3'

3'-dTdTTCGACGUAGUUACCUCGUGUAUGUC-5'

103. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 22

5'-GCAUCAAUGGAGCACAUACAGGGAdTdT-3'

3'-dTdTTCGUAGUUACCUCGUGUAUGUCCCU-5'

104. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 23

5'-UUAAGCUGCAUCAAUGGAGCACAUdTdT-3'

3'-dTdTAAUUCGACGUAGUUACCUCGUGUA-5'

105. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 24

5'-GCUGCAUCAAUGGAGCACAUUU-3'

3'-UUCGACGUAGUUACCUCGUGUAU-5'

106. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 25

5'-AAGCUGCAUCA AUGGAGCACAUU-3'

3'-UUUUCGACGUAGUU ACCUCGUGU-5'

107. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 26

5'-GCAUCA AUGGAGCACAUACAGUU-3'

3'-UUCGUAGUU ACCUCGUGUAUGUC-5'

108. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 27

5'-CUGCAUCA AUGGAGCACAUACUU-3'

3'-UUGACGUAGUU ACCUCGUGUAUG-5'

109. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 28

5'-AAGCUGCAUCA AUGGAGCACAUACUU-3'

3'-UUUUCGACGUAGUU ACCUCGUGUAUG-5'

110. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 29

5'-GCUGCAUCA AUGGAGCACAUACAGUU-3'

3'-UUCGACGUAGUU ACCUCGUGUAUGUC-5'

111. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 30

5'-GCAUCAAUGGAGCACAUACAGGGAUU-3'

3'-UUCGUAGUUACCUCGUGUAUGUCCCU-5'

112. The composition of claim 39, wherein the siRNA comprises the following sequence:

SEQ ID NO: 31

5'-UUAAGCUGCAUCAAUGGAGCACAUUU-3'

3'-UUAUUCGACGUAGUUACCUCGUGUA-5'

113. The composition of any of claims 1-112, comprising a plurality of active agents.

114. The composition of claim 113, wherein at least two active agents comprise nucleic acids.

115. The composition of claim 114, wherein at least one of said nucleic acids is a siRNA species.

116. The composition of claim 114, wherein at least one of said nucleic acids is an oligonucleotide.

117. The composition of any of claims 114-116, wherein a first and a second nucleic acids target separate genetic sequences.

118. The composition of claim 117, wherein the separate genetic sequences are found in separate genes.

119. The composition of claim 117, wherein the separate genetic sequences are found in the same gene.

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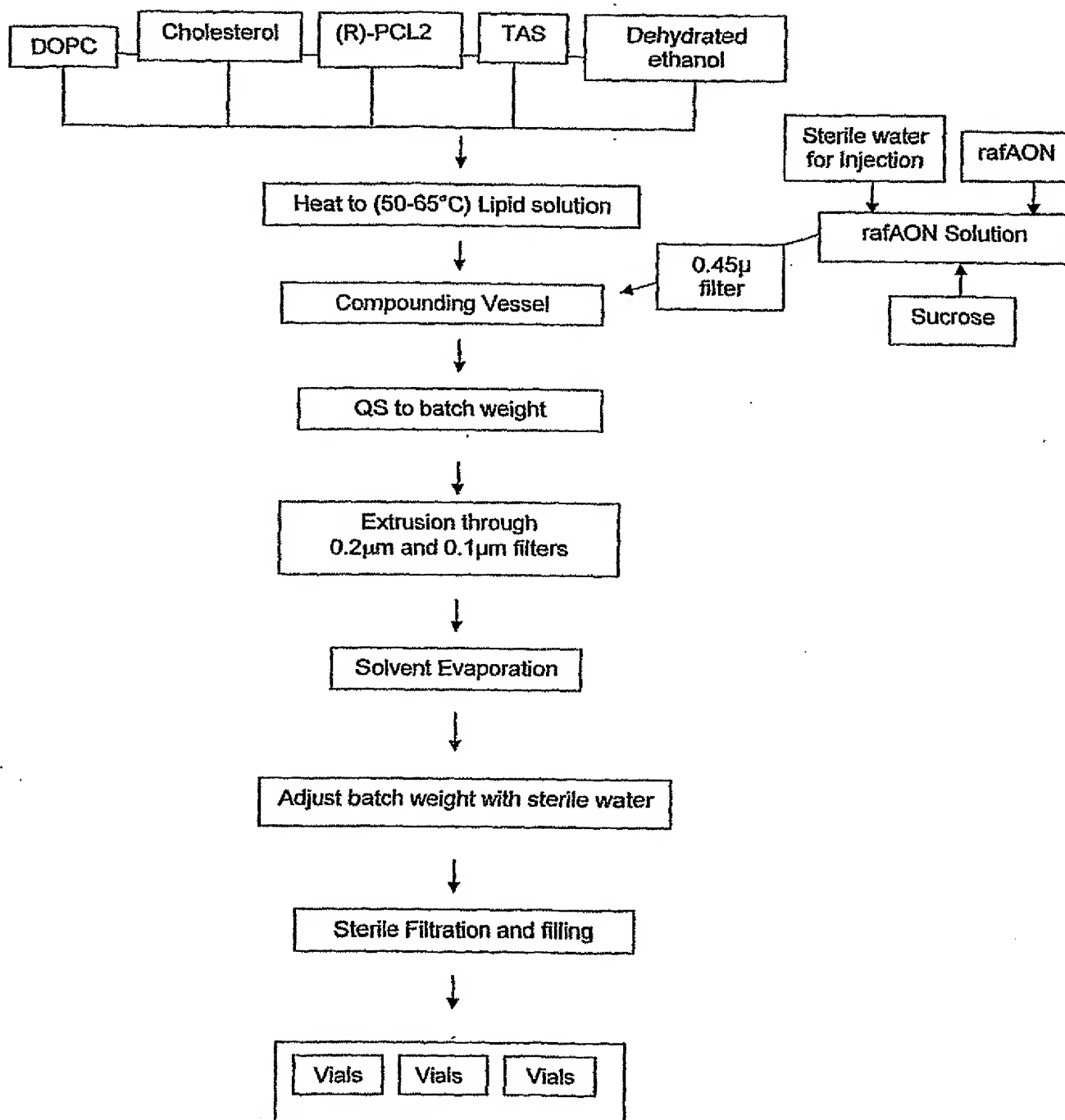


Fig. 1

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**Therapeutic Efficacy of DDAB-Liposome or Cationic Cardiophilin Liposome-Based Antisense Oligonucleotide (AON) in combination with Taxotere® against Human Prostate (PC-3) Tumor Growth in SCID Mice\***

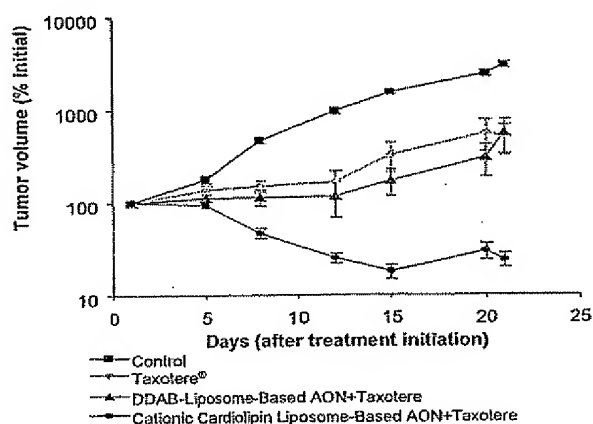
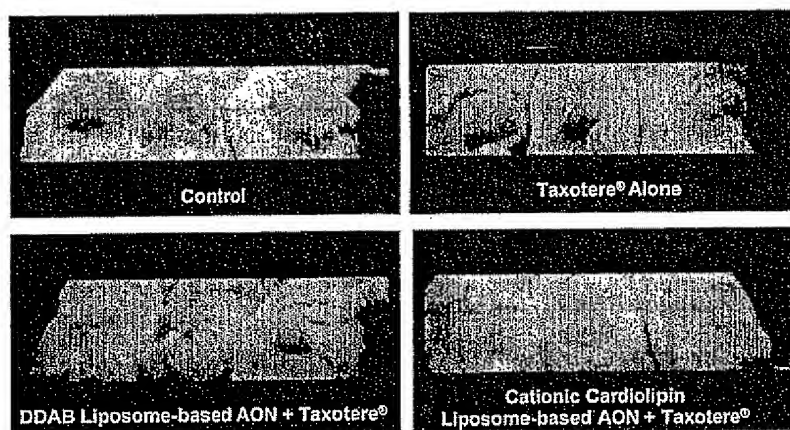


Figure 4A

**Treatment**  
 DDAB Liposome or Cationic Cardiophilin Liposome-Based AON Formulations (25 mg/kg, BID X 5)  
 Taxotere®: Day 2, (10 mg/kg), Day 5, (5 mg/kg)

\*Obtained from Harlan Sprague Dawley, Indianapolis, IN

**Effect of DDAB-Liposome or Cationic Cardiophilin Liposome-Based Antisense Oligonucleotide (AON) in Combination with Taxotere® Against Human Prostate (PC-3) Tumor in SCID Mice**



**Treatment**  
 DDAB or Cationic Cardiophilin Liposome-based AON (12.5 mg/kg, BID x 5)  
 Taxotere® Day 2 (10 mg/kg), Day 5 (5 mg/kg)

Figure 4B



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**Toxicity of DDAB\*-Liposome or Cationic  
Cardiolipin Liposome-Based Antisense  
Oligonucleotide (AON) in Mice<sup>†</sup>**

Test Article**	Dose (mg/kg/day)***	Number of Mice	%Survival Male	%Survival Female
DDAB Liposome- Based AON	35	10	0	60
Cationic Cardiolipin Liposome-Based AON	35	10	90****	100

\*Dimethyldioctadecyl ammonium bromide

\*\*Control- 100% Survival

\*\*\*17.5 mg/kg, BID X 5

\*\*\*\*Animal died due to technical error

<sup>†</sup> Obtained from Charles River, Wilmington, MA

Figure 4C

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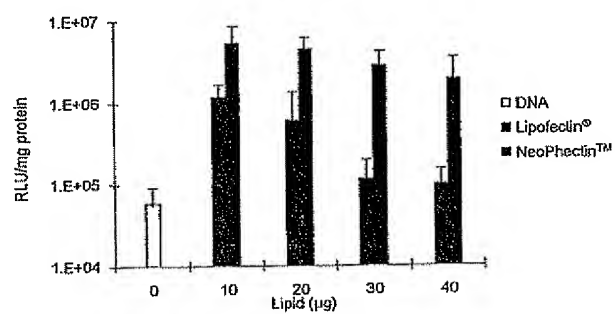


Figure 5 A

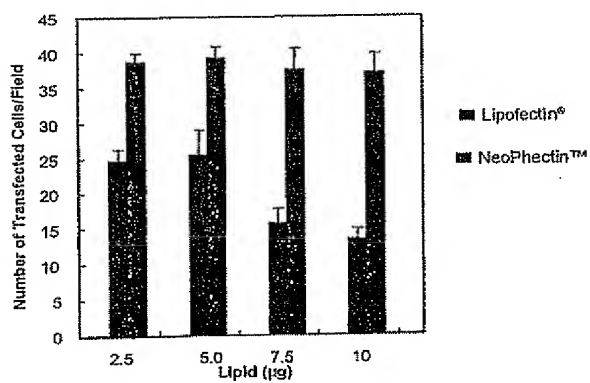


Figure 5 B

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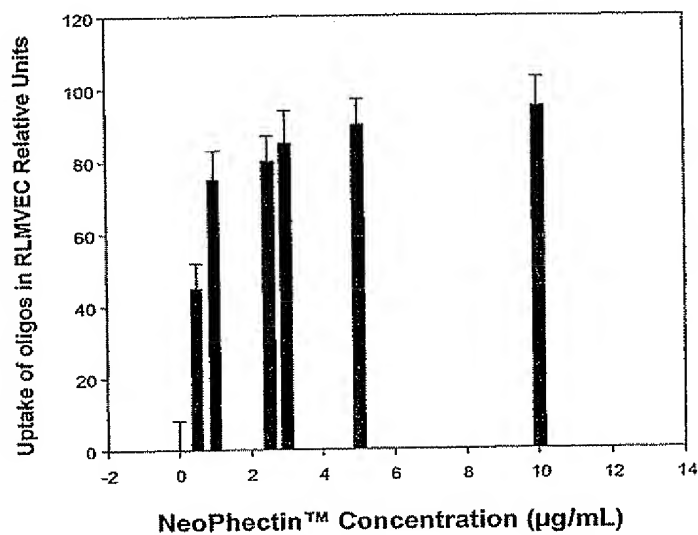


Figure 5 C

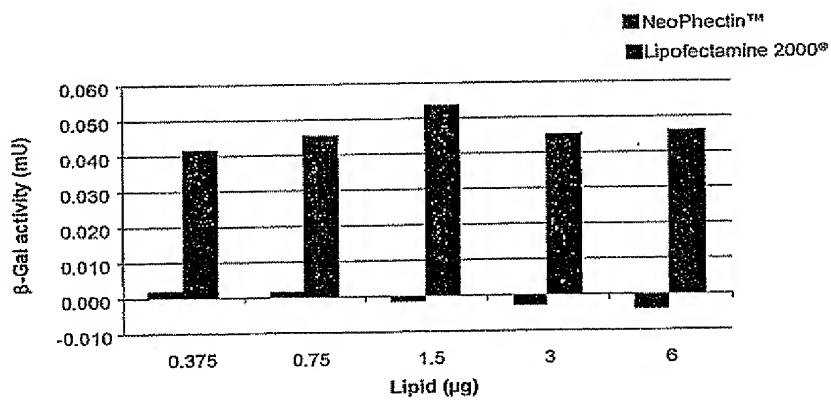


Figure 5 D

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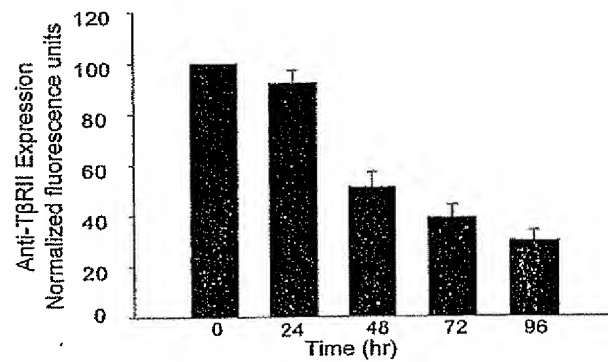


Figure 5 E

**Effect of NeoPhectin™ Mediated Delivery of RNAi (200nM) Oligonucleotides In Primary Cells After 48 Hours\***

**Control**

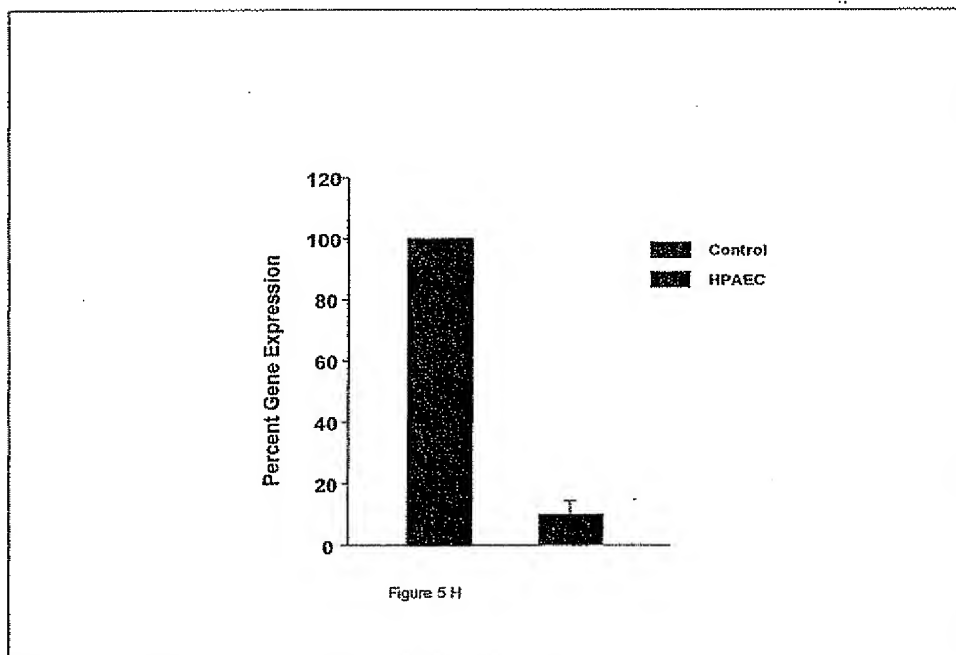
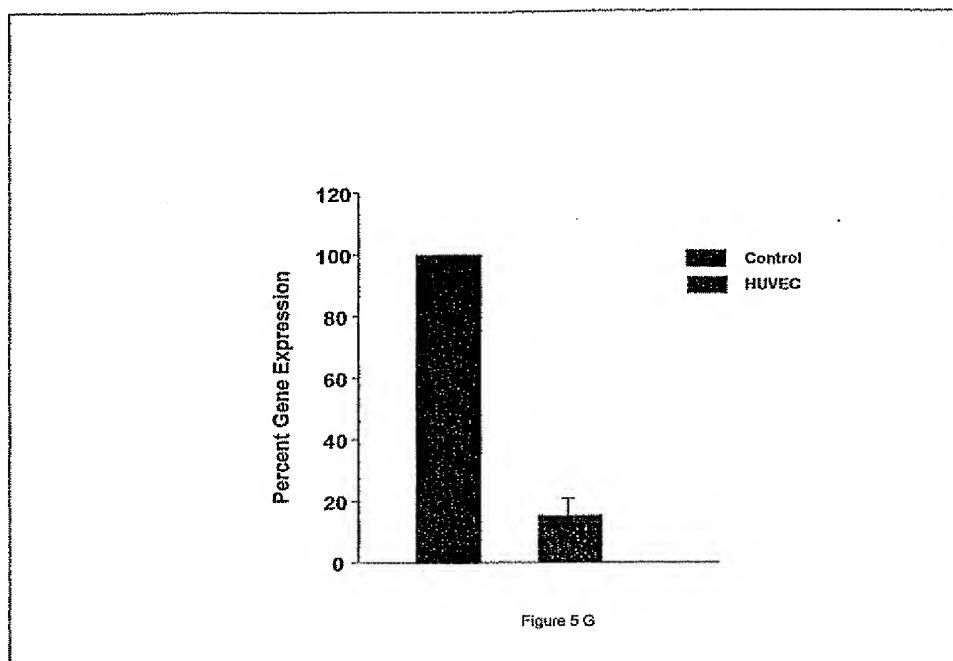


**NeoPhectin**



Figure 5 F

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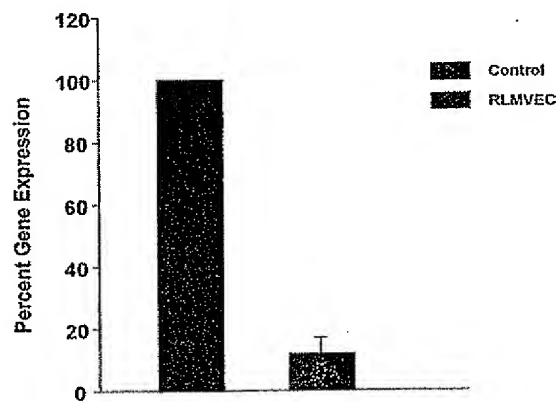


Figure 51

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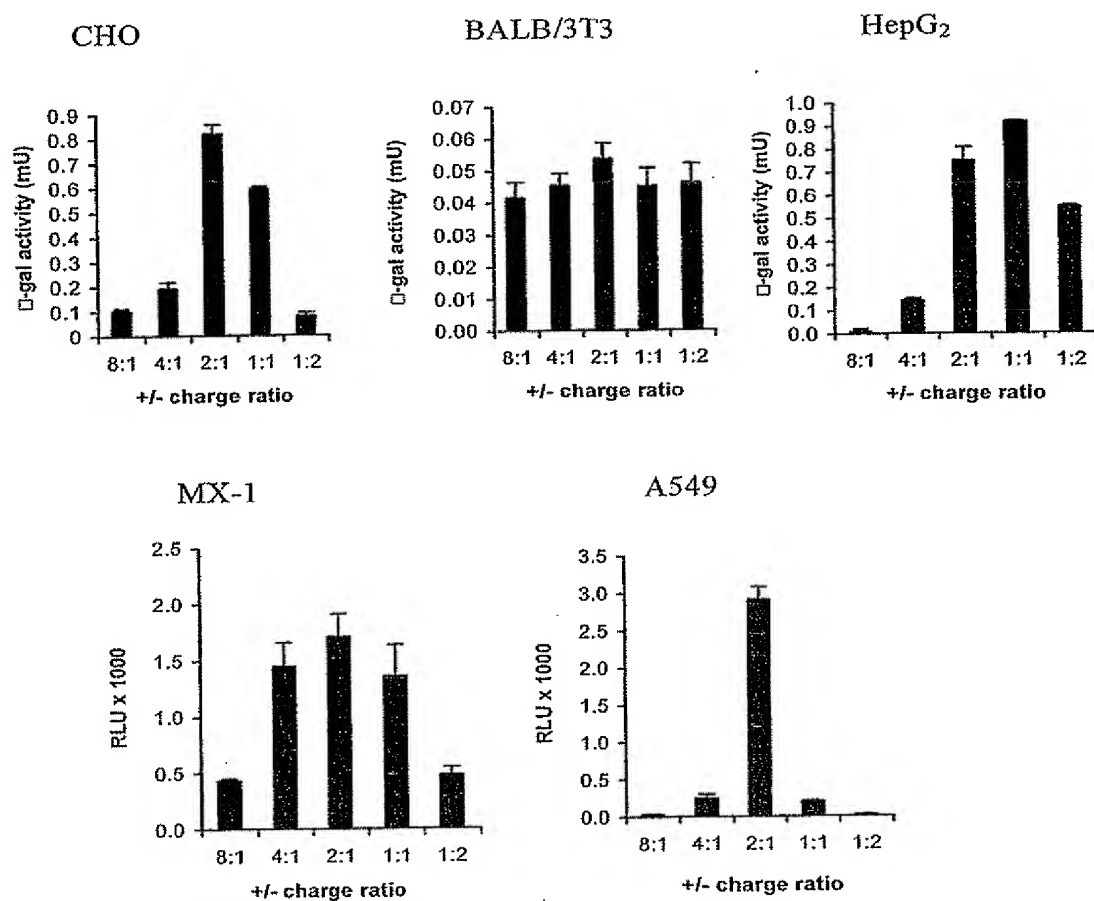


Figure 6A

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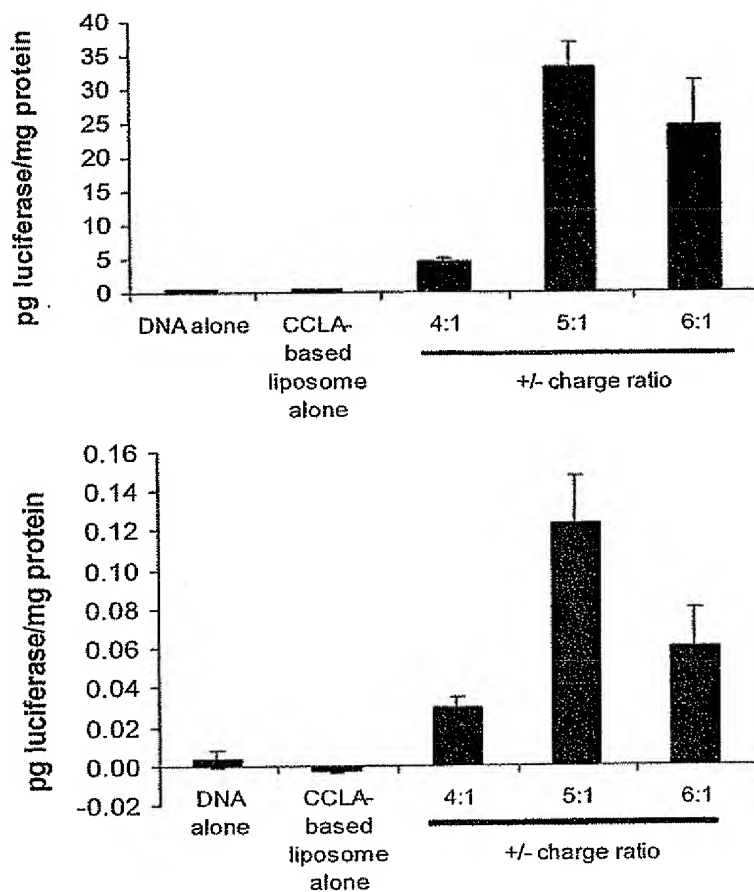


Figure 6B

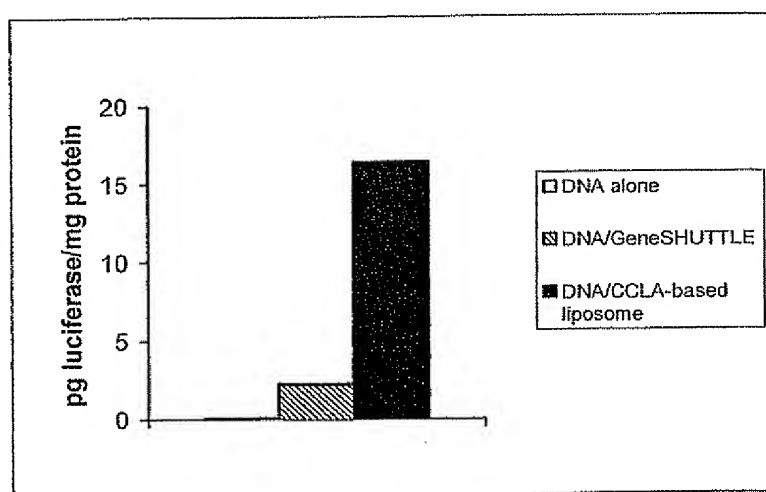


Figure 6C



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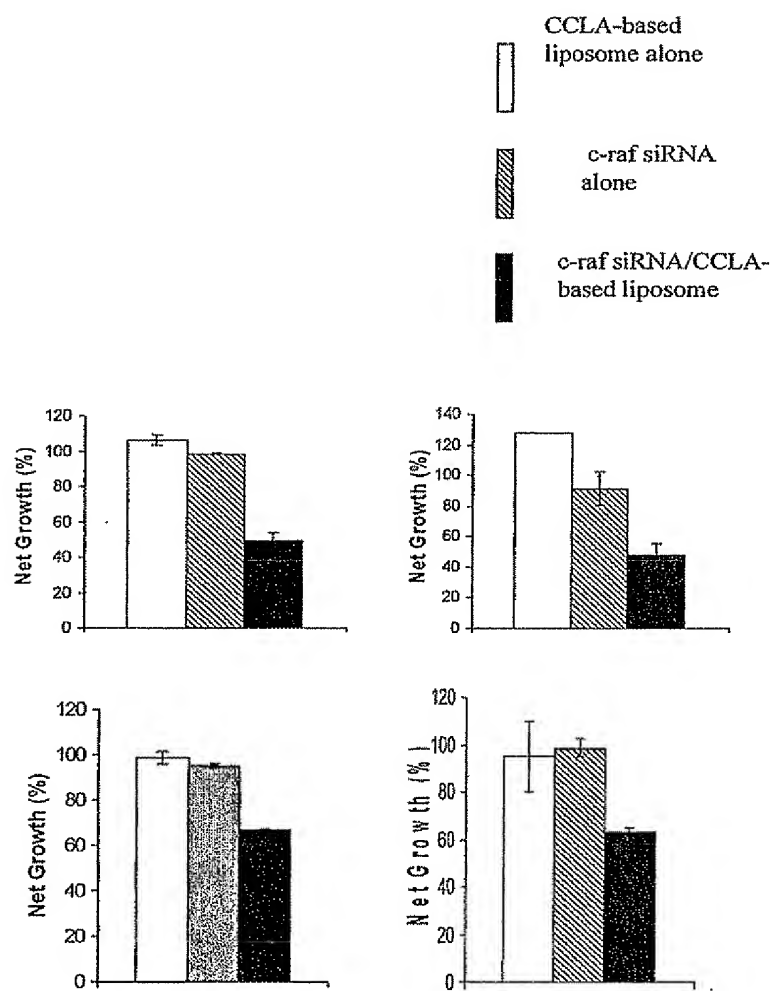


Figure 6D

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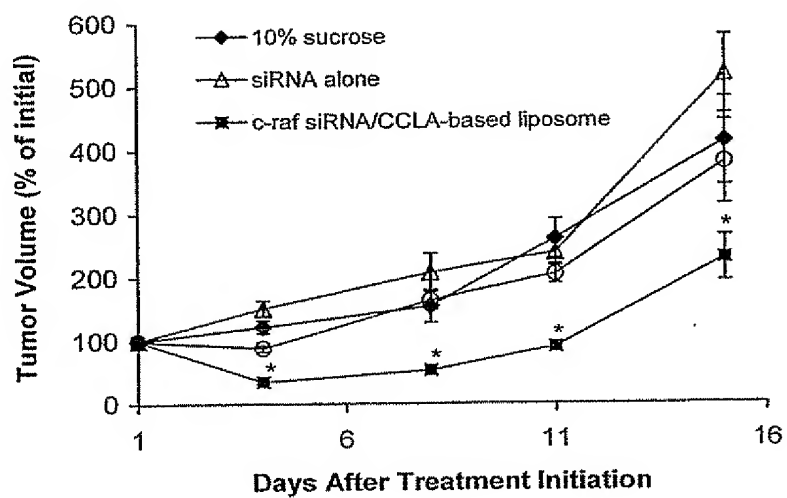


Figure 6E

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Figure 7A

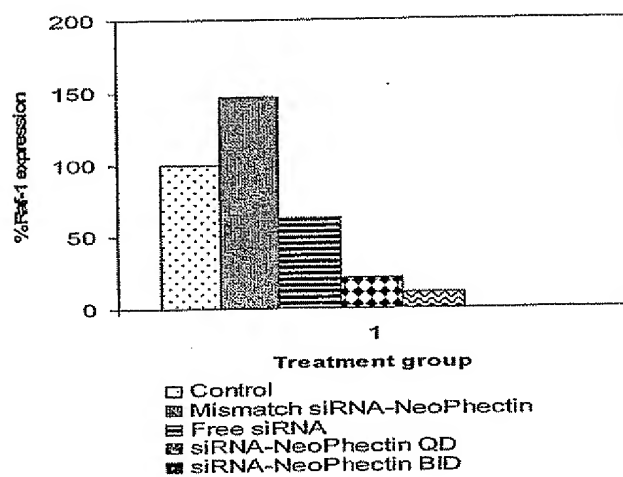
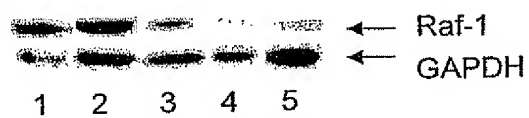


Figure 7B

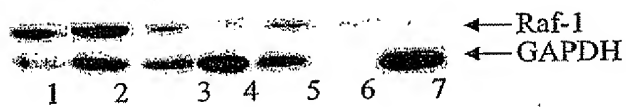


Figure 7C

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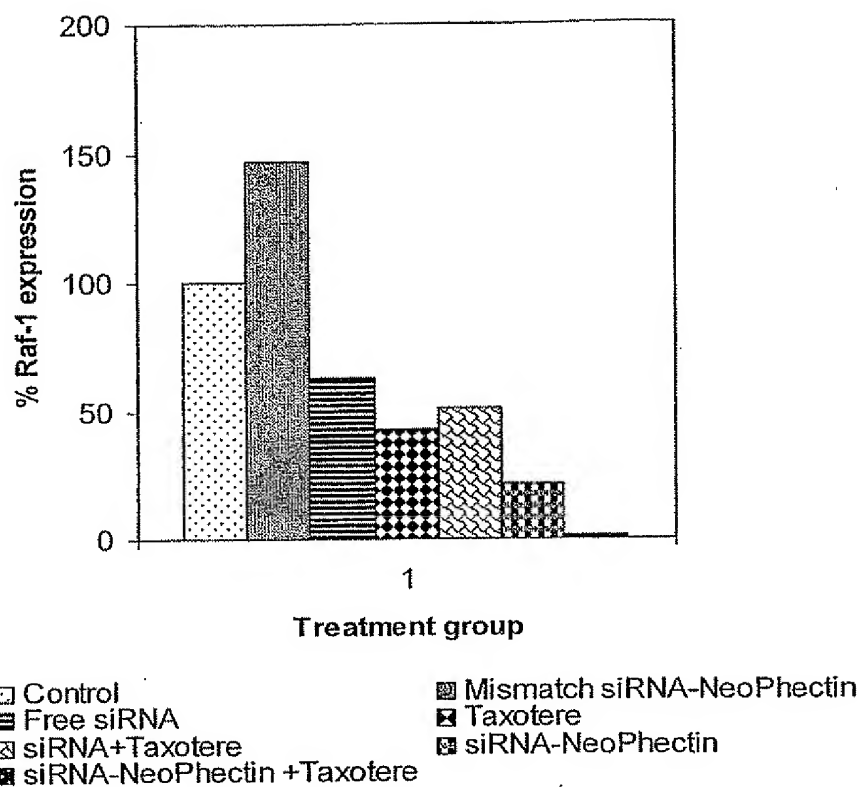


Figure 7D

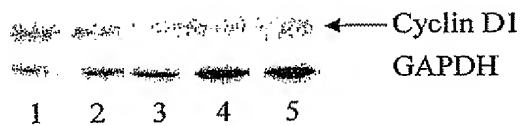


Figure 7E

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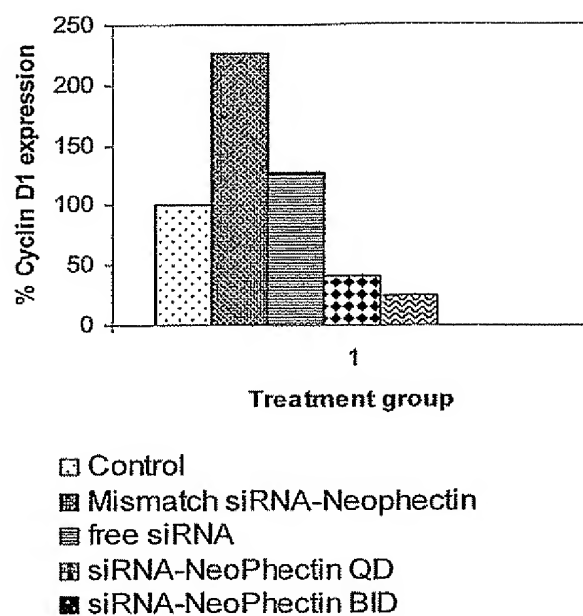


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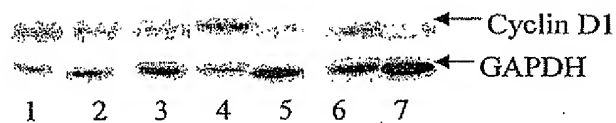


Figure 7G

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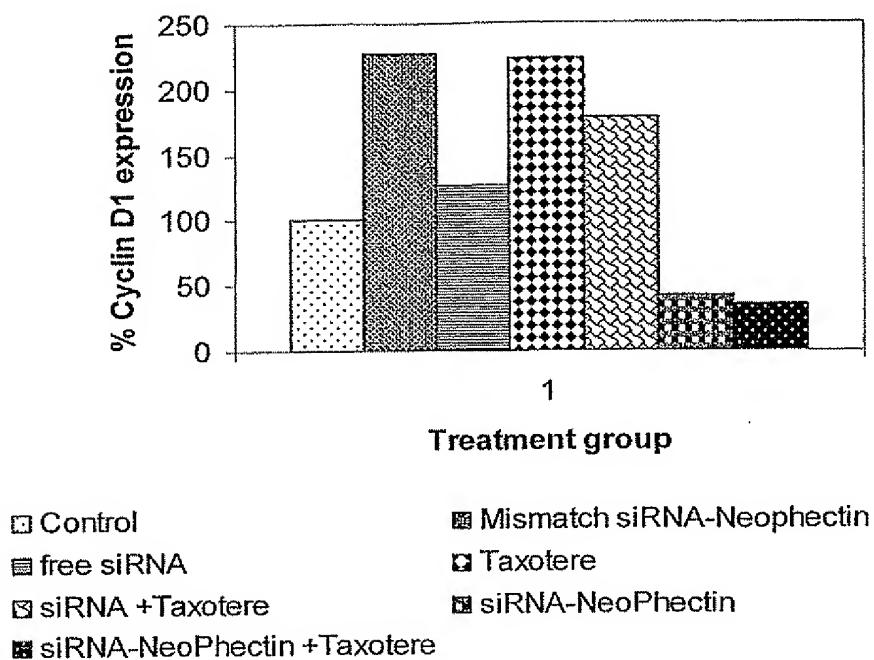


Figure 7H

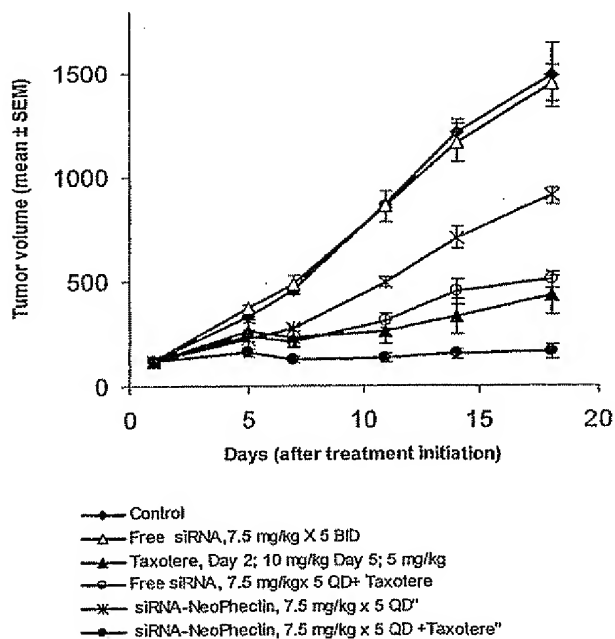
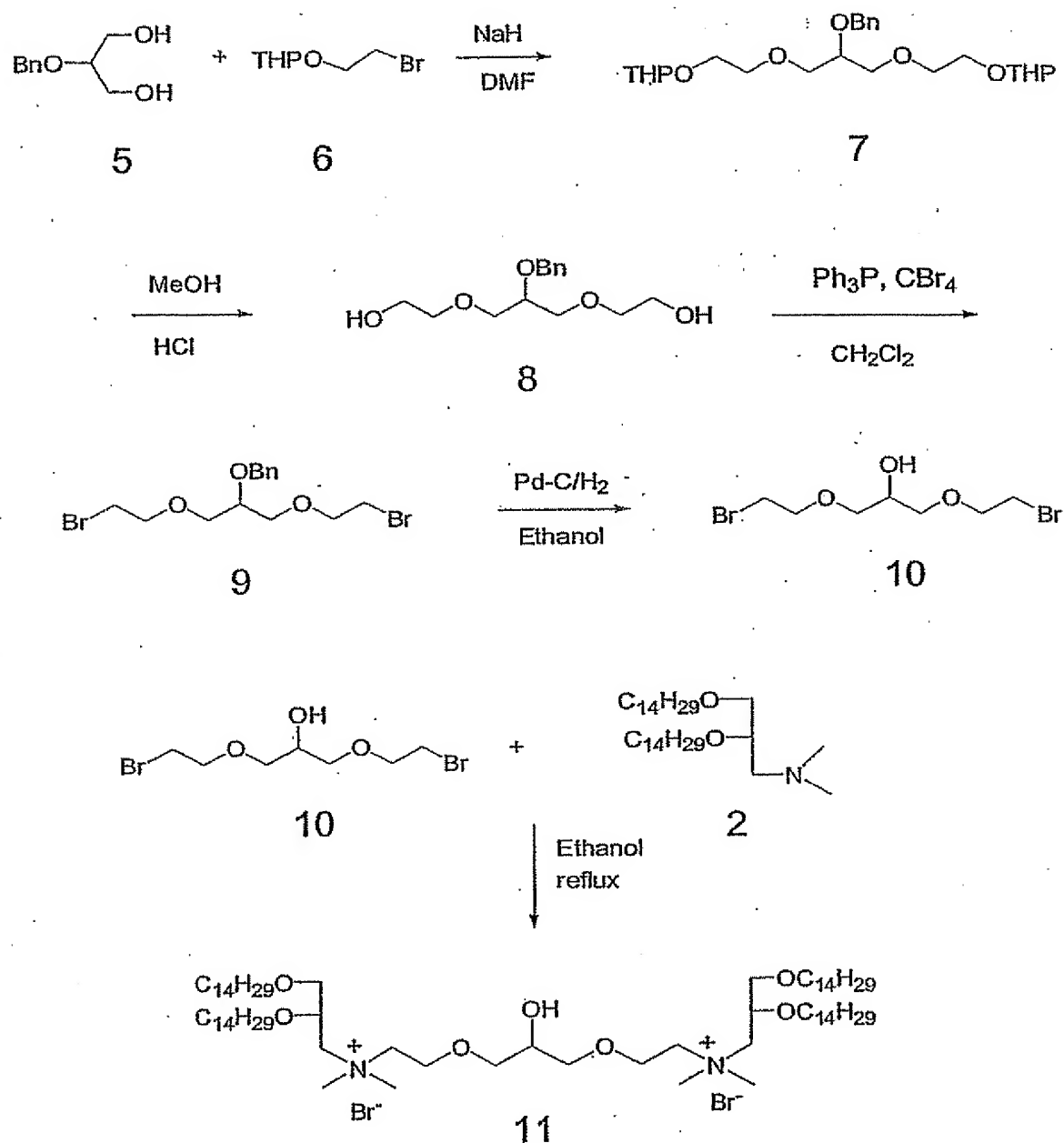


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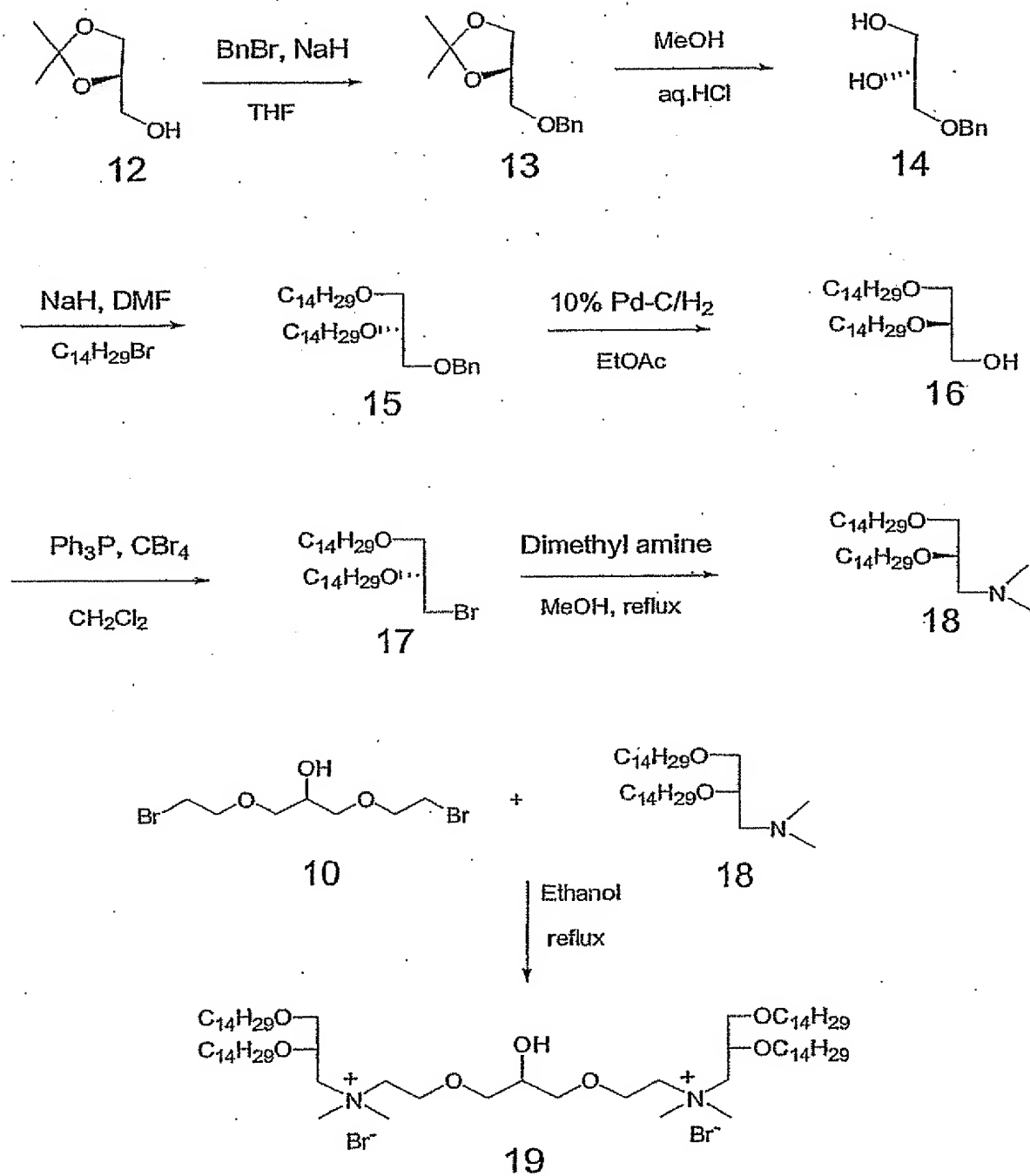
17/19

Figure 8A



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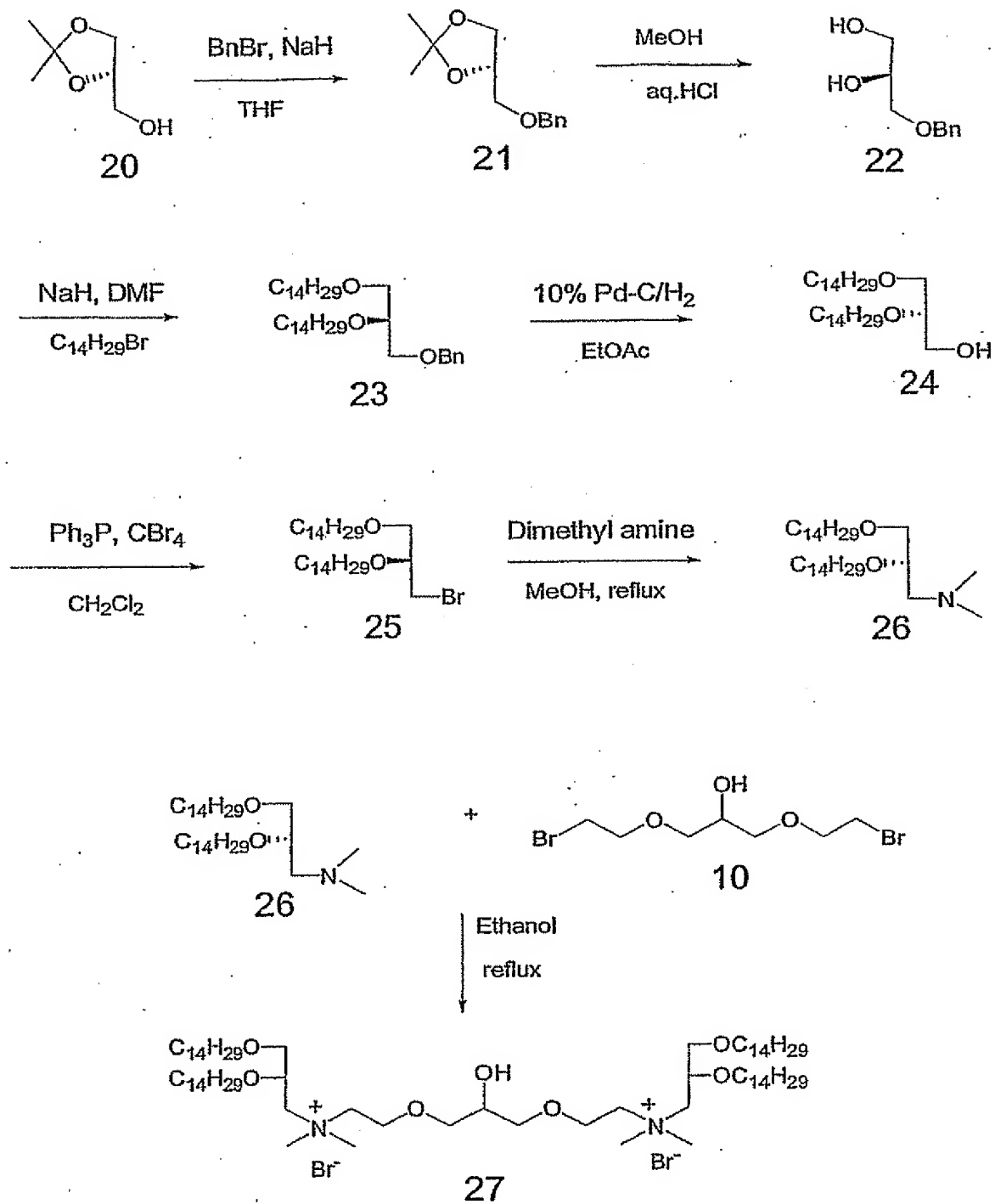
Figure 8B





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Figure 8C



232362.ST25

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 JAMIL, Haris  
 ZHANG, Jia-Ai  
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